MECHANISMS THAT CONTROL FOLLICULAR DOMINANCE IN CATTLE

By

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To my parents Francisco and Maria Antonia (Toña),

my brother, Francisco Javier

my sisters Ana Maria Flor Alba Yuritza and Elsa and

my late uncle, Juan

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MECHANISMS THAT CONTROL FOLLICULAR DOMINANCE IN CATTLE

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A series of experiments examined potential mechanisms that control follicular dominance in cattle On day 5 of the estrous cycle ablation of the first wave dominant follicle with an injection of human Chorionic Gonadotropin (hCG 3 000 IU) resulted in ovulation of the follicle formation of an accessory corpus luteum and higher concentrations of plasma progesterone in treated heifers. The second wave dominant follicle emerged earlier in hCG-treated heifers and all treated heifers had three follicular waves Treatment with hCG initiated turnover of the dominant follicle and altered subsequent follicular dynamics A second experiment tested the effect of follicle-stimulating hormone-pituitary (FSH-P) injections beginning at day 12 on folliculogenesis and ovulatory responses in the presence or absence of a first wave persistent dominant follicle Follicle recruitment and induction of class 2 (6 to 9 mm)

and class 3 (>9 mm) follicles were delayed during the superinduction period in the presence of a persistant dominant follicle. However, no differences were detected between groups in the final number of ovulatory follicles, corpus luteum and recovered embryos. Inhibitory effects of a persistent dominant follicle were overridden with continuous injections of FSH. A granulosa cell culture system was developed to test the effects of intra- and interovarian regulators (inhibin forms) on granulosa cell secretion of estradiol. Purified porcine FSH (CY-FSH) stimulated estradiol secretion of granulosa cells between 72 to 96 h of culture. Also evaluated were effects of different fractions of follicular fluid (follicular fluid complete, follicular fluid fraction without inhibins and an inhibin-enriched fraction) from day 5 dominant and day 12 atretic dominant follicles on estradiol secretion of granulosa cells. A 10 ng/ml concentration of CY-FSH stimulated estradiol secretion compared to 0.5 ng/ml. Fractions of follicular fluid without inhibins and enriched in inhibins from day 5 dominant follicles decreased estradiol secretion. In contrast, follicular fluid complete and follicular fluid without inhibins from day 12 atretic dominant follicles inhibited estradiol secretion but the enriched fraction of inhibins stimulated granulosa cells to secrete estradiol. Collectively, the series of *in vivo* and *in vitro* experiments documented that the dominant follicle exerts both interovarian and intrafollicular affects on follicle function in cattle.

CHAPTER 1
INTRODUCTION

On the basis of gross and histological studies of ovaries, it was proposed in 1960 that two waves of follicular activity occurred during the bovine estrous cycle (Rajakoski 1960). After measuring follicles and quantifying steroids in blood and follicular fluid, Ireland and Roche (1983) concluded that there were three follicular waves during the estrous cycle, and each wave resulted in the formation of a dominant follicle. In 1984, Pierson and Ginther characterized bovine ovarian folliculogenesis, for antral follicles ≥ 3 mm by repeated daily ultrasound measurement and concluded that heifers had estrous cycles with two follicular waves. However, others (Fortune et al., 1988; Savio et al., 1988; Sirois and Fortune, 1988) reported three waves of follicular development during the estrous cycle. Research in this area is motivated by the desire to solve the long-time mystery involving the mechanisms underlying recruitment, selection and development of follicular dominance that is characteristic of monovulatory species (Ginther et al., 1996).

In general, two processes lead to development of the normal species-specific number of ovulatory follicles. First, follicle recruitment results in the development of a pool or cohort of follicles from which the preovulatory follicle emerges as a

result of follicle selection The follicle becomes dominant and continues development towards ovulation, while others regress (Fortune et al 1991) This pattern of dominant follicle development also occurs during the luteal phase of the estrous cycle in which dominant follicles undergo atresia instead of ovulation The phenomenon of dominance is clearly of interest because mechanisms of follicular dominance must be subverted to successfully superovulate domestic animals or humans Furthermore an understanding of dominant follicle development and dominance is important to the development of reproductive control system to synchronize ovulation in dairy cattle

At present, little is known about mechanisms by which a follicle attains and maintains dominance (Fortune 1993) To study follicular dominance differences between morphological and functional dominance have to be established Morphological dominance is defined as the largest developing follicle on either ovary The functionally dominant follicle appears to have the ability to inhibit growth of smaller follicles and can ovulate under appropriate hormonal conditions (Fortune 1993)

Basic research is needed to elucidate the mechanisms controlling dominance during follicular development in cattle to optimize the techniques to control reproductive cycles in cattle Objectives of the present research were a) to characterize alterations in ovarian follicular and corpus luteum dynamics after injection of hCG on day 5 of the estrous cycle b) to evaluate the effect of follicle stimulating hormone-pituitary (FSH-P) on superinduction of follicles and ovulatory responses in the presence or absence of an active-first wave persistent dominant

follicle and to elucidate the role of inhibins as intraovarian regulator of estradiol secretion during different periods of follicular dominance The last objective require development of an *in vitro* system to examine regulation of estradiol secretion by bovine antral granulosa cells

CHAPTER 2
LITERATURE REVIEW

Ovarian Follicular Growth and Development

Reproductive cyclicity in the female is maintained by endocrine paracrine and autocrine interactions. The central role of pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH) is to direct ovarian follicular recruitment, growth atresia and ovulation (Richards and Hedin 1988). The ovary has two main functions: production of the oocyte, the female gamete, and to provide the hormonal environment to sustain reproductive function through the production of steroid hormones such as progesterone (P_4), estradiol-17ß (E_2) and other regulatory factors. Gonadotropin secretion influences, among other things, development of follicles in the ovary. The factor or factors that determine the early stages of follicular growth in the ovary at a pre-determined time are unknown. The fate of a follicle could be to remain quiescent, to begin development but later become atretic or to mature and ovulate. In mammals the number of primordial follicles present in the ovaries is fixed at birth or in the immediate post-natal period depending on the species. In the cow, between 40 000 to 80 000 quiescent primordial follicles can be found at birth, but this number is reduced to approximately 3 000 by 15 to 20 years of age (Erickson, 1966). Since 99% of the primordial follicles fail to ovulate during

the lifespan of an animal development of an ovulatory follicle is a rare biological event and folliculogenesis is a complex process (Ireland, 1987)

Early Stages of Follicular Development

The initial stages of folliculogenesis occur independently of gonadotropic hormones Antral follicles initially become responsive to FSH and then are dependent on FSH for sustained growth Inhibins activin, insulin like growth factor I (IGF-I) and their binding proteins have direct and indirect effects on granulosa and theca cells that can modulate follicular development and steroidogenesis (Roche, 1996)

Ginsburg et al (1990) demonstrated that primordial germ cells in the mouse appear by day 7 post coitum in the extra embryonic mesoderm posterior to the primitive streak. These cells migrate to the gonad by day 12 post coitum. Transforming growth factor-β_1 (TGF-β_1) produced by the gonad appears to be one of the factors responsible for the chemotropic attraction of primordial germ cells (Godin and Wylie 1991)

According to Hilscher (1991) female germ cells undergo only one proliferative wave of oogonial divisions whereas gametogenesis in the male involves two proliferative waves In the bovine ovary onset of meiosis occurs at approximately day 70 of embryonic life (Erickson 1966) However, meiosis is not completed in the embryonic ovary, and germ cells are arrested in the diplotene stage of prophase I Conversion of oogonia into primary oocytes depends on contact with cells derived from the *rete ovarii* The first germ cells to enter meiosis

are located at the inner border of the ovary establishing a two-way communication between germ and somatic cells The first follicles are formed around day 70 of pregnancy in the ewe and day 130 of pregnancy in the cow (Marsana et al , 1991)

The majority of mammals restrict oogonial proliferation to the prenatal period of development (e g. pigs and ruminants) or to shortly after birth during the early neonatal period (e g , rodents and rabbits Van den Hurk et al 1997) Oogonia are transformed into primary oocytes characterized by a prolonged meiotic prophase and surrounded by a layer of flattened pregranulosa cells When a full layer of cuboidal granulosa cells is acquired around the oocyte the follicle becomes an intermediary and then a primary follicle These primordial intermediary and primary follicles constitute the resting stockpile of nongrowing follicles located at the periphery of the ovarian cortex and form the bulk of follicles contained in the ovary (Driancourt et al 1983b) These follicles are progressively depleted during the reproductive life span Initiation of ovarian function in the cow occurs during fetal development Primordial follicles start to grow in response to an unknown trigger (Webb et al 1992) when they continue development until ovulation or atresia The primordial follicle possesses an oocyte surrounded by a single granulosa layer consisting of 14-28 flattened granulosa cells in cattle which is in turn encompassed by a basement membrane (Van den Hurk et al 1997) It has been reported that initiation of follicle growth is not dependent on gonadotropins It has been estimated that it takes approximately 6 months for a primordial follicle to develop into a large dominant follicle in sheep (Cahill and Mauléon 1980) In the cow the estimated

time for a follicle to grow from 0 13 to 8 56 mm is approximately 41 5 days the equivalent of two estrous cycle (Lussier et al 1987)

One of the most critical steps in folliculogenesis is the transformation of primordial follicles into primary follicles The growth of a follicle appears to begin with enlargement of the primary oocyte the proliferation of surrounding granulosa cells and the organization of thecal cells external to the basement membrane (Paton and Collins 1992) The nature of the signal to convert flattened pregranulosa cells into a cuboidal epithelium is not clear However the oocyte provides cues to this conversion (Greenwald and Roy 1994) By a series of mitotic divisions the unilaminar primary follicle is converted into a multilayer preantral secondary follicle Each primordial follicle contains a single layer of pregranulosa cells resting on a basal lamina and radially arranged around an oocyte which is arrested in diplotene stage of meiosis (Paton and Collins 1992) Interstitial cells surround the follicle in immediate proximity to the basal laminae and start to differentiate into theca cells when follicular growth starts During the early stage of a secondary follicle connective tissue fibers are arranged parallel to the basement membrane underneath the granulosa to form a thecal layer At the same time a glycoprotein coat the zona pellucida is formed between the growing oocyte and the innermost granulosa layer At the end of this stage hormone producing large epithelioid cells and a capillary network additionally constitute the theca (Van den Hurk et al 1997) With the appearance of an antral cavity the secondary follicle is converted into a tertiary follicle It is frequently stated that gonadotropic hormones are not necessary for early growth of follicles and that FSH and LH

become indispensable for further development only at the transformation of the secondary to the tertiary follicle. The relative constant number of preantral follicles throughout the estrous cycle of the mouse is evidence that cyclic changes in gonadotropins do not affect the pool of preantral follicles. However in the rat, rhesus monkey and hamster the number and responsiveness of preantral follicles to gonadotropins do change during the estrous cycle. In the human ovary long-term follicular growth is severely impaired in the absence of gonadotropins (Greenwald and Roy, 1994). In the hypophysectomized ewe follicular development is arrested at a stage between 2 to 3 mm in diameter (McNatty et al. 1990).

Schematically, antral follicular development involves two phases. In the first phase, early antral follicles grow slowly (up to 2 mm in sheep and 3 - 4 mm in the cow) and follicular growth rate is related to the proliferation rate of granulosa cells and is not really dependent on gonadotropin supply. The second phase or growth is dependent on gonadotropin secretion. This phase is characterized by rapid follicular growth, mainly due to enlargement of the antrum, and corresponds to terminal development of antral follicles up to the preovulatory stage. During this phase the steroidogenic capacity and the responsiveness of granulosa cells to FSH and LH increases (Monniaux et al., 1997). It has been shown (Adams et al. 1992b) in domestic ruminants that terminal follicular growth is associated with fluctuations of FSH concentrations, which suggests a stimulating effect of FSH on emergence of each follicular wave.

Goodman and Hodgen (1983) suggested the terms of recruitment, selection and dominance to describe the development of antral follicles. Recruitment is a

gonadotropin-dependent event during which a group of follicles gain the ability to respond to gonadotropin and develop dependence on them for continued growth Selection is a process whereby only a few of the recruited follicles are selected to escape atresia and survive to ovulate In cattle selection is defined as the time when an estrogen-active follicle promotes its growth and inhibits the growth of other follicles (Sunderland et al , 1994) Factors other than size and E_2 concentrations such as P_4 may be important for establishing which follicle becomes dominant during the selection phase (Sunderland et al 1994) Xu et al (1995b) suggest that selection of the dominant follicle may be a passive process in which the first follicle that acquires LH receptors in its granulosa cells is selected to become the dominant follicle The acquisition of LH receptors in granulosa cells will enable these follicles to respond to LH in addition to FSH whose concentrations in blood have declined to basal levels at the time of selection (Adams et al 1992b) Dominance is the mechanism that an ovulatory follicle uses to escape atresia and inhibits recruitment of a new cohort of follicles Expression of LH receptor in granulosa cells is associated with dominance (Xu et al 1995a Yuen et al , 1996)

Ovarian Follicular Dynamics During the Bovine Estrous Cycle

Antral follicle development was considered originally to be a continuous state of turnover without a distinct pattern of follicular growth regression and atresia (Marion et al 1968) A classical study of Rajakoski (1960) along with studies by Matton et al (1981) indicated that at least two periods of turnover of antral follicles occur during the estrous cycle of the cow Later during the 1980's daily ultrasound

scanning of ovaries confirmed that generally two, but sometimes only one nonovulatory follicle wave occurs in the luteal phase of the cycle before final development of the ovulatory dominant follicle after luteal regression (Pierson and Ginther, 1984; Fortune et al., 1988; Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a; Lucy et al., 1992; Sunderland et al., 1994; Roche, 1996).

Unlike primates (Goodman and Hodgen, 1983), non-ovulatory follicles in cattle develop during the early- and mid-luteal phase of the estrous cycle (Rajakoski, 1960; Ireland, 1987). This is reflected by the increase in E_2 concentrations in blood a few days after ovulation and during the mid-luteal phase. Each follicular wave is characterized by a simultaneous emergence of a group of 5 to 7 follicles (> 5 mm in diameter) from the pool of small follicles. One from this group rapidly emerges and grows larger than the others in the cohort and is considered the "dominant" (Fortune, 1993), while the others become atretic and regress. The dominant follicle normally reaches a maximum size of 10 to 15 mm in diameter and remains dominant for a period of 5 to 7 days, until it becomes atretic and regresses in size. The regressing dominant follicle is replaced with a new dominant follicle grown from the next wave of follicles. If luteal regression occurs during the growth phase or early period of dominance, then the dominant follicle, free from the restrictive hormonal milieu imposed by P_4 secretion from the corpus luteum upon the hypothalamus and pituitary gland, will continue to develop to preovulatory size and trigger the hormonal cascade leading to ovulation (Webb et al., 1992).

Follicle growth is a process during which the follicle progressively acquires a number of properties each of which is an essential prerequisite for further development. Failure to acquire these properties at the correct time and in an exact sequence will lead to failure of the process and to deterioration of the follicle through atresia (Campbell et al., 1995). Antrum formation occurs at a follicular diameter (fixed ovaries) of 0.14-0.26 mm in cattle and it takes 40 days for follicles to reach ovulatory size (Lussier et al., 1987). In cattle there is a marked hierarchy in the antral follicle population with a large number (20-30 follicles of 3-4 mm in diameter) of gonadotropin responsive follicles (follicles that can respond to gonadotropin stimulation, but do not need gonadotropins) a few (1-4 follicles of > 4-5 mm) gonadotropin-dependant follicles (follicles that need gonadotropins to further develop) and one ovulatory follicle (Campbell et al., 1995).

Follicular dynamics can be defined as the process of continual growth and regression of antral follicles that leads to the development of the preovulatory follicle (Lucy et al., 1992). Ireland (1987) hypothesized that the turnover of dominant follicles during the estrous cycle is regulated by the differential response of selected and unselected follicles in the cohort to alterations in patterns of secretion of gonadotropins which in turn result in a differential production of intrafollicular stimulatory or inhibitory factors that control selection, dominance and atresia. Analysis of patterns of development of large follicles in mammalian species shows that large follicles do not develop at random, but their development occurs only during particular reproductive states and/or during particular times of the reproductive cycle (Fortune, 1994). This pattern of follicle development is

associated with follicular changes in expression of mRNA encoding gonadotropin receptors (Xu et al., 1995a) and steroidogenic enzymes (Xu et al., 1995b) that allow selected follicles, when exposed to the requisite hormonal environment, to develop and ovulate in response to the preovulatory gonadotropin surge. Endocrine signals such as gonadotropins, inhibin, and steroids, as well as locally produced growth factors, such as insulin-like growth factor I (IGF-I), transforming growth factor α (TGF-α), transforming growth factor ß (TGF-ß), epidermal growth factor (EGF), and other peptide hormones such as activin and follistatin, are responsible for the control and coordination of these process (Armstrong and Webb, 1997).

Early during the estrous cycle a cohort of follicles is recruited out of the pool of smaller antral follicles (2 to 4 mm). Recruitment is not a random or isolated phenomenon. Follicles seem to be recruited as groups or cohorts, suggesting that they have received a signal that allows them to continue growth and development rather than regress (Fortune, 1994). The mechanism that controls recruitment of these small follicles and determines which follicles are recruited is unknown. The signal that stimulates recruitment appears to be a slight elevation in plasma FSH (Fortune, 1994). Xu et al. (1995a) hypothesized that changes in expression of mRNA for FSH and LH receptors may be important for recruitment of a cohort of follicles and selection and atresia of the dominant follicle in cattle. However, the steady state of FSH receptor mRNA level in healthy follicles did not correlate with follicle size, nor did the level of FSH receptor mRNA change with stage of the first follicular wave. Adams et al. (1992b) reported that 2 to 4 days before a new wave of follicle development there is an increase in FSH, which suggests that increases

in circulating concentrations of FSH initiate the emergence phase for dominant follicle growth. The number of follicles recruited is usually greater than the typical number of ovulatory follicles for a given species. However, only a species-specific number of ovulatory follicles continues to grow for more than a few days and reaches ovulatory size (Fortune, 1994).

After 2 to 4 days of recruitment (days 2 to 4 of the estrous cycle), several medium-sized follicles (6 to 9 mm) can be detected by ultrasonography. This is the phase of selection in which a single follicle emerges from the pool of recruited follicles and continues to grow, whereas other recruited follicles decrease in size (Lucy et al., 1992). Sunderland et al. (1994), using ultrasound analysis and the ratio of E_2 P_4 concentrations in follicular fluid, reported that days 1 to 3 of the estrous cycle are the selection phase for development of the early diestrous first wave dominant nonovulatory follicle. Ginther et al. (1996) defined time of deviation (or selection) as the beginning of the greatest difference in growth between the two largest follicles in the ovary. They indicate that the term deviation of follicles is a major event in the selection process, and the terms deviation and selection are synonymous. Moreover, deviation was characterized by a slower rate of growth for 1 to 4 days before the subordinate follicle attained maximum diameter and by the immediate cessation of growth of the largest subordinate follicle (Ginther et al., 1996; 1997).

The completion of the selection phase is defined in the cow as the time when an estrogen-active follicle promotes its own growth and inhibits the growth of other follicles (Sunderland et al., 1994). Adams et al. (1993b) showed that the decrease

In circulating concentrations of FSH is an integral component of the selection mechanism Exogenous FSH given for 2 days at the expected time of selection delayed selection as indicated by a 1 5 day delay in significant divergence of the growth profiles of the dominant and first subordinate follicles There was greater growth and delayed regression of the first and second subordinate follicles The same treatment regimen given after selection did not alter follicular development and atresia However it is not known how the FSH decline exerts its effect on selection Perhaps selection involves competition among the cohort of follicles for utilization of FSH whereby the most successful follicle becomes dominant This competition becomes acute when FSH levels decline Results from Xu et al (1995a, 1995b) suggest that selection of the dominant follicle may be a passive process in which the first follicle that acquires LH receptors in its granulosa cells is selected to become the dominant follicle The acquisition of LH receptors in granulosa cells will enable these follicles to respond to LH in addition to FSH Indeed concentrations of FSH in blood have declined to basal levels at the time of selection (Adams et al 1992b)

On approximately day 5 growth of usually only one follicle is sustained while growth rate of other follicles declines This first wave dominant follicle remains dominant from day 5 to ≈ day 9 (Driancourt et al 1991a) and during this period of follicular dominance no new follicles > 5 mm are detected within the ovaries (Lucy et al 1992 Fortune 1993) Therefore, a dominant follicle is defined as a large ovarian follicle (> 10 mm) that is recruited and selected during a follicular wave and regulates growth of other follicles on the ovary (Lucy et al 1992) The dominant

follicle would somehow cause demise of follicles of the same cohort and would also suppress a new wave of follicular development. At present little is known about the mechanisms by which a follicle attains and maintains dominance (Fortune, 1993). Perhaps a follicle is selected for dominance because it is in the right stage of development and is better able to respond to the slight elevation of and the subsequent decline in FSH to continue its growth (Fortune 1994). The ability of the dominant follicle to continue growth and development in an environment of lower levels of FSH may be due to increased blood flow and/or to the acquisition of LH receptors by the granulosa cells (Zeleznick, 1993). Xu et al (1995b) showed that theca interna cells of dominant follicles collected on day 4 after initiation of the first follicular wave had the highest levels of mRNAs for $P450_{scc}$ and P450c17 20 lyase which ensure that the theca interna cells of these follicles are capable of producing large amounts of androgen substrates for E_2-17β biosynthesis in the granulosa cells. The acquisition of LH receptors in granulosa cells may be critical to the establishment and maintenance of follicular dominance, whereas FSH receptors may only play a permissive role (Xu et al 1995a). Large antral follicles can transfer their gonadotropic requirements from FSH to LH. This transition in gonadotropic requirement from FSH to LH is the probable mechanism whereby the preovulatory follicle can withstand the fall in FSH that occurs at the onset of the follicular phase following luteal regression (Campbell et al 1995).

The follicle selected for dominance from each wave not only continues to grow, but also differentiates functionally in ways that prepare it for ovulation. The secretion of increased quantities of E_2 by the selected follicle appears to be of

primary importance and sets it apart from its sister subordinate follicles. Feedback regulators such as E_2 and inhibin produced by the dominant follicle (or perhaps by the whole cohort of follicles during the first few days after recruitment) cause a decrease in FSH concentrations that will not even support the further growth of subordinate follicles (Fortune, 1994).

It is important to distinguish between morphological and functional dominance. Morphological dominance is characterized by size of the follicle with the largest follicle present on a pair of ovaries being defined as morphologically dominant. Functional dominance appears to have two aspects: ability of the dominant follicle to inhibit the growth of smaller follicles and the capability to ovulate under appropriate hormonal conditions (Fortune, 1993). The largest follicle of the first and second wave of cows with three waves is by definition morphologically dominant (Fortune et al., 1991; Fortune, 1993). The first wave dominant follicle at day 12, despite being the largest follicle, had lost its functional dominance as early as day 10 based on emergence of a new wave of follicular development (Sunderland et al., 1994). These results and others (Savio et al., 1990a) show that morphological dominance of a follicle persists much longer than functional dominance. Dinancourt et al. (1991) reported that dominance is characterized by the large difference in diameter between the largest and the second largest follicles and the decrease in the number of follicles smaller than 8 mm.

Two hypotheses have been postulated to explain how the dominant follicle exerts dominance. One hypothesis states that the dominant follicle secretes a product that directly impairs further growth and development of subordinate follicles.

In monotocous species like cattle such a factor would have to be endocrine in nature, since it would induce regression of subordinate follicles on both ovaries. The other hypothesis states that the dominant follicle could cause the regression of subordinate follicles indirectly, via negative feedback mechanisms in which E_2 and inhibin would cause a decrease in FSH concentrations that would not support further growth of subordinate follicles (Fortuna 1994). A third hypothesis which explains dominance from an intraovarian point of view, states that production of local ovarian factors such as the insulin-like growth factor (IGF) system components of the transforming growth factor β superfamily, fibroblast growth factor, and the epidermal growth factor/transforming growth factor α family can inhibit the development of subordinate follicles directly (Campbell et al. 1995; Armstrong and Webb, 1997).

Sunderland et al. (1994) indicated that the period of days 1 to 3 of the estrous cycle is the selection phase for development of the early diestrous dominant nonovulatory follicle, whereas days 10 to 12 is boyh the selection phase for development of the next dominant follicle and the period when the first dominant follicle ceases to function or loses its dominance, becomes estrogen-inactive and begins to undergo atresia. In two-wave cycles, maturation of the second dominant follicle coincides with spontaneous regression of the corpus luteum, and the follicle ovulates after luteolysis. Alternatively, the second wave dominant follicle may become atretic and a third follicular wave will be initiated (Lucy et al., 1992) and becomes the ovulatory follicle.

Sirois and Fortune (1988) reported that follicular waves during the estrous cycle occur at approximately 7 day intervals. For three-wave cycles the first, second and third waves start on days 2, 9 and 16, respectively; for two-wave cycles the second wave started about 2 days later than the average for animals with three waves (day 11; Fortune et al., 1991). Waves of follicular development in cattle occur regularly during both the estrous cycle and pregnancy. During the estrous cycle, the preovulatory gonadotropin surge perturbs the pattern of regular waves by triggering an abrupt ovulation of the follicle that is functionally dominant at the time of estrus.

More than 99% of ovarian follicles undergo the degenerative process of atresia during reproductive life (Hsueh et al., 1994). If the ovulatory signal is absent, the mature follicle undergoes degeneration. It has been shown that the dominant follicle of the first wave, which normally undergoes atresia, can ovulate provided that the ovulatory stimulus is given before it reaches the advanced regression stage of development (Savio et al., 1990a; Sirois and Fortune, 1990).

In antral bovine follicles > 1 mm in diameter, the earliest and most prominent feature of atresia is death of granulosa cells (presence of pycnotic nuclei) that leads to almost total destruction of the granulosa cell layer lining the inner follicle wall (Rajakoski, 1960). Atresia of the dominant nonovulatory follicle is characterized by a significant decrease in the number of granulosa cells, a decrease in both LH and FSH receptors (Ireland and Roche, 1983), and a diminished capacity to produce E_2 between days 7 and 13 of the estrous cycle (Badinga et al., 1992; de la Sota, 1995). The factors involved in regulation of follicular atresia are not clear, but it has been

demonstrated that decreasing the LH pulse frequency to a luteal phase secretory pattern results in faster atresia of the dominant follicle (Sirois and Fortune 1990) Thus the dominant follicle may avoid atresia when exposed to a higher LH pulse frequency during the follicular phase Induction of a low progesterone environment (< 2 ng/ml) results in increased growth or size of the dominant follicle higher plasma concentrations of E_2 and a greater lifespan or persistence (Savio et al 1993a) In addition to sustained follicular growth functional dominance also was observed as there was a suppression in the number of other follicles > 4 mm in diameter (Savio et al 1993a) Stock and Fortune (1993) reported that slight increases in LH pulse frequency promoted prolonged follicular growth and dominance associated with increased plasma E_2 They suggested that the demise of nonovulatory dominant follicles during normal estrous cycles occurs through feedback effects of luteal progesterone, which maintains a low LH pulse frequency and E_2 production

Based mainly on morphological criteria atresia of antral follicles can be divided into several stages (Hsueh et al 1994) Stage I is characterized by a small number of granulosa cells (< 10%) with pyknotic nuclei close to the follicular cavity; however some of the granulosa cells are still undergoing mitosis Stage II shows many pyknotic granulosa cells (10-30%) few cells in mitosis and cell debris in the follicular cavity The basement membrane loses its integrity and there is a leukocytic infiltration into granulosa cell layers In the oocyte meiotic-like changes are evident Stage III is characterized by a reduction in granulosa cell number an absence of mitosis and collapse of the follicle. The oocyte also undergoes germinal vesical breakdown (Hsueh et al 1994)

Fate of the thecal cell layer during atresia varies among different species. In the human, rat, and rabbit, theca cells undergo extensive hypertrophy during follicular atresia. In contrast, hamster follicles do not exhibit marked morphological changes despite sharply reduced follicular vascularity and total collapse of the granulosa layer. In the sheep, theca cells undergo nuclear condensation and degeneration similar to those observed in granulosa cells (Hsueh et al., 1994).

Biochemical changes also occur during atresia. These changes include reduced DNA synthesis of granulosa cells, suppressed expression of connexin, a gap junction protein, decreased gonadotropin binding, decreased expression of mRNAs for aromatase and gonadotropins receptors, and decreased E_2 synthesis concomitant with increased progesterone production. The latter change appears to be due to both a decrease in $C_{17,20}$-lyase activity, that leads to a decrease in androgen substrate for granulosa cell aromatization, and a loss of aromatase activity (Hsueh et al., 1994).

During atresia, there is an increase in expression of several genes, including the IGF-binding proteins (IGFBP), which is related to a deprivation of endogenous IGF-I that is essential for survival of follicle cells (Hsueh et al., 1994). Recent biochemical evidence has demonstrated apoptosis of granulosa cells during follicular atresia in bovine ovaries (Jolly et al., 1994). These authors suggested that apoptotic death of granulosa cells in the cow may also occur in healthy follicles during the luteal phase of the estrous cycle and/or occur early in the atresia process before other morphological or biochemical signs of degeneration or dysfunction are

evident. Thus a variable incidence of apoptotic cell death may occur at different stages of follicular development.

Structure and Function of the Antral Follicle

The primary function of the mammalian ovarian follicle is the release of an oocyte capable of being fertilized by sperm. This involves growth and maturation of the follicle as well as enlargement, ovulation, and meiotic division of the egg (Richards, 1980). The identification of the ovarian follicle occurred in 1672 by Regnier de Graaf, and mature preovulatory follicles are named Graafian follicles. It was not until 1827 that Karl Ernst von Baer discovered that the mammalian egg was a tiny part of the entire follicular structure. In the early 1900's the gonads as well as the pituitary were recognized as organs of internal secretion (Richards, 1980).

A single follicle consists of a diplotene oocyte and associated somatic cells surrounded with a basal lamina. Small follicles have a single layer of follicular cells and form the largest number in the ovary. The ovarian follicle consists of outer layers of theca cells which encircle inner layers of granulosa cells, which are separated from blood vessels and theca cells by a basement membrane lining the follicle. Inner layers of granulosa cells surrounding the oocyte are designated as the oocyte-cumulus cell complex (Amsterdam et al., 1989). Follicular growth ends with ovulation or atresia. The growth of the follicle is ensured by the growth of the oocyte, follicular cell proliferation, and enlargement of the antrum (Mariana et al., 1991). Follicles appear to begin growth under all physiological conditions, including

pregnancy ovulation or periods of anovulation (Richards 1980 Amsterdam and Rotmensch 1987) The number of follicles beginning to grow each day changes throughout life and appears related primarily to the number of follicles in the nongrowing or resting pool The larger the pool the greater the number of follicles beginning to grow With age as follicles continuously leave the pool of nongrowing follicles the number of follicles in the resting pool becomes drastically reduced and ultimately is exhausted This reduction is associated with a decrease in the number of follicles that begin to grow and eventually ovulate (Richards 1980)

The first sign of growth is the resumption of cell proliferation by the squamous granulosa cells an increase in the size of the oocyte and a change in shape of the granulosa cells (Hirshfield 1991) In the adult ovary folliculogenesis starts when follicles leave the pool of resting follicles to enter the growth phase From there the early growing follicle undergoes a developmental process including a dramatic course of cellular proliferation and differentiation (Gougeon, 1996) The oocyte and the follicle grow in a biphasic manner During the first phase, which ends shortly before antrum formation growth of oocyte and granulosa cells is linearly and positively correlated The second phase consists of enhanced mitotic activities of granulosa and thecal cells and an increase in volume of follicular fluid (Greenwald and Roy, 1994) The oocyte enlarges rapidly during early follicular growth and reaches full size early in the developmental process (Hirshfield 1991) Later in development the growth of the oocyte is slow compared to growth of the follicle Growth of the oocyte involves accumulation of proteins either through direct synthesis within the oocyte or by transfer from the antrum During the active phase

of oocyte growth, content of proteins is increased 100-fold and RNA content enhanced 20-fold RNA synthesis is increased in the nucleus of the growing oocyte compared to that found in the primordial oocyte Synthetic activity decreases steadily in oocytes from follicles with an antrum (Menana et al 1991)

The follicle acquires several distinctive morphological features during growth theca interna (steroidogenic cells) and theca externa (connective tissue cells) which form the outer layers of the follicle a fluid-filled antral cavity a capillary network and the thick acellular zona pellucida surrounding the oocyte (Hirshfield 1991) As the follicle enters its final phase of growth, fluid-filled spaces appear between the granulosa cells which soon coalesce into a large single fluid-filled antral cavity The accumulating fluid appears to be formed by filtration of thecal blood The composition of follicular fluid differs considerably from plasma with lower glucose and lipid concentrations in follicular fluid There are also differences in amino acid concentrations (Hirshfield, 1991)

The Granulosa Cell Compartment

The ovarian follicle has the unique capacity to change in structure and function to influence the cyclic processes of female reproduction A finely tuned process of differentiation occurs in all constituents of the follicle during folliculogenesis Granulosa cells play an obligatory role in creating the conditions necessary to resume oogenesis ovulation fertilization and implantation (Paton and Collins 1992) Granulosa cells display a high degree of structural change and play a key role in the functional maturation of the entire follicle

The early development of the female gonad is characterized by the migration of a mitotically active cohort of germ cells from the region of the embryonic hindgut and endoderm of the yolk sac to the gonadal ridge. As germ cells enter meiosis granulosa cells seem to arise from the *rete ovarii* or from mesonephric tubules or mesenchymal cells in the gonadal anlage and they organize around the primary oocytes (Amsterdam and Rotmensch 1987). The development of the steroidogenic potential in differentiating granulosa cells consists of early and late events (Amsterdam *et al*., 1989). The early events are related to mobilization of cholesterol reorganization of endosomes lysosomes lipid droplets and particularly mitochondria and smooth endoplasmic reticulum, which are the carriers of membrane-bound enzymes converting cholesterol to a variety of steroid hormones. Late events consist of stable changes in cell shape, intercellular communication through gap junction formation and development of organelles associated with steroidogenesis (Amsterdam et al., 1989).

Granulosa cells from the various layers within the same follicle display morphological heterogeneity suggesting the existence of a differentiation gradient in the follicle. Cells adjacent to the follicular basement membrane contain a higher density of LH receptors than cells located in the inner layers of the pre-ovulatory follicle (Amsterdam et al., 1989). The granulosa cell layer surrounding the oocyte called the corona radiata, produces elongated cytoplasmic processes which traverse the zona pellucida towards the oocyte and form gap junctions at the area of contact. Due to the nature of a nonvascular environment of granulosa cells they need a way of intercellular communication between neighboring cells. In addition

to a complex paracrine system of membrane-soluble hormones there is also a direct cell-to-cell communication via gap junctions (Amsterdam and Rotmensch 1987). Gap junctions are cellular structures between adjacent cells, wherein the apposed cellular membranes are separated by an apparent gap of approximately 3 nm. Gap junctions are formed by connexins. They allow communication or electrical coupling between adjacent cells that can be open or closed. Gap junctions are composed of two symmetrical structures (connexons) that create an intracellular channel that allows passage of ions and small molecules from cell to cell (Grazul-Bilska et al., 1997). Also adherance junctions have been described in granulosa cells. They are characterized by association of filamentous dense material on the intracellular aspect of the plasma membrane (Amsterdam and Rotmensch, 1987). Gap junctions allow ions and small water soluble molecules to pass directly from the cytoplasm of one cell to the cytoplasm of another, thereby coupling the cells both electrically and metabolically (Amsterdam et al., 1989; Paton and Collins, 1992). Gap junctions are absent in the primordial follicle, where desmosomes and adherance junctions are the dominant structures comprising the junctional complex (Amsterdam and Rotmensch, 1987; Amsterdam et al., 1989). Gap junctions appear during differentiation of the antral follicle and parallel the onset of gonadotrophic action on the follicle (Amsterdam and Rotmensch, 1987; Amsterdam et al., 1989; Paton and Collins, 1992). Molecules of 1200 daltons and smaller (e.g., inorganic ions, amino acids, nucleotides and sugars) can pass through the junctional channels and move from cell to cell. For example, cyclic AMP (cAMP) has been identified as a possible mediator of biological function

through gap junctions The functional significance of gap junctions between granulosa cells may lie in propagating cAMP-mediated events such as the response to FSH or LH, thereby allowing the signal to reach the interior of the avascular preovulatory follicle (Amsterdam and Rotmensch, 1987) Changes in the degree of dispersion of granulosa cells during follicular maturation seem to be related to quantitative changes in intercellular junctions Biochemical differentiation of granulosa cells is associated with gap junction formation in response to FSH stimulation Morphological studies suggest that cAMP might be associated with changes in cell shape and organization of the cytoskeleton (Ben-Ze'ev and Amsterdam 1987)

Granulosa cells display a high degree of structural change during the differentiation process There are alterations in cellular shape and size in plasma membranes and in internal membranes of the endoplasmic reticulum mitochondrial cristae Intercellular junctions and cytoskeleton. Granulosa cells are flat and epithelioid in the earliest stage of development When proliferation starts the flattened layer of granulosa cells becomes a multilayered stratified epithelium consisting of cuboidal cells (Paton and Collins 1992) There is also a change in the type of intracellular organelles Immature granulosa cells contain mainly a rough endoplasmic reticulum, which reflects a high level of protein synthesis required for growth and proliferation Smooth endoplasmic reticulum increases its development when steroidogenic potential increases (Paton and Collins 1992) Complex tubular mitochondrial cristae is typical of FSH stimulated cells whereas a lamellar type is observed in immature cells (Amsterdam and Rotmensch 1987)

The cytoskeleton mediates many of the structural alterations that coincide with differentiation-dependent changes in the metabolic activity of granulosa cells (Paton and Collins 1992). Cytoskeletal elements are believed to be involved in aggregation and internalization of ligand-bound gonadotropin receptors (Amsterdam and Rotmensch 1987). These elements provide the machinery for moving organelles from one place to another when granulosa cells are exposed to gonadotropins and movement of substrate among organelles also needs to be facilitated to enhance steroid synthesis (Paton and Collins 1992). The regulation of granulosa cell differentiation at the level of the cytoskeleton seems likely to prove as complex as other intraovarian regulatory mechanisms. One important mechanism by which organization of cytoskeleton can be regulated is by the activity of transmembrane proteins which link cytoskeletal proteins to the extracellular matrix. The extracellular matrix is an important regulator of differentiation of granulosa cells (Paton and Collins, 1992).

Granulosa cells are circumscribed by two layers of insoluble extracellular material. The peripheral cells border the basal membrane of the follicle and corona radiata cells attach to the zona pellucida (Amsterdam and Rotmensch 1987). Zona pellucida glycoproteins are synthesized by the growing oocyte (Driancourt et al 1993b). It is unclear to what extent granulosa cells contribute to the formation of these layers, however, their interaction with the extracellular matrix components appears to affect differentiation events resulting in varying biochemical and structural characteristics.

A gradient of differentiation exists in granulosa cells in relation to their distance from the basal membrane of the follicle. Subpopulations of granulosa cells exist in ovarian follicles (Amsterdam et al., 1988). In the rat, there exists evidence of morphological and functional differences between antral cells in close proximity to the antral cavity and mural cells in close proximity to the basement membrane. They differ relative to number of gap junctions (Wiesen and Midgley, 1993), FSH receptors (Monniaux and De Reviers, 1989) and in the mitotic index (Hirshfield, 1992). Likewise, basal steroid production is lower in mural than in antral bovine granulosa cells cultured in the absence of FSH (Roberts and Echternkamp, 1984). Although morphological similarities were noticed between bovine antral and mural granulosa cells in response to FSH stimulation, the steroidogenic responses to FSH of antral granulosa cells in terms of E_2, P_4, and testosterone production are considerably higher than those of mural granulosa cells (Rouillier et al., 1996). There is a higher content of LH receptors, mitochondrial P-450 side chain cleavage enzyme and 3ß-hydroxysteroid dehydrogenase in the peripheral granulosa cell layers (in apposition to basal membrane) than in the inner layers. Also, cells close to the basal membrane exhibit a decreased proliferative activity, which suggests an active role for the basement membrane in the induction and maintenance of granulosa cell differentiation (Amsterdam and Rotmensch, 1987).

Extracellular matrix is associated with changes in cell shape and contact which are accompanied by simultaneous alterations in organization and expression of cytoskeletal proteins. Gonadotropins and extracellular matrix may induce granulosa cell differentiation in a coordinated fashion by their effect on cell shape

cell contact intercellular communication and expression of cytoskeletal elements (Amsterdam and Rotmensch, 1987)

Fibronectin is present in the follicle as a component of the basal lamina and as a soluble fraction of follicular fluid which increases with increased follicle size (Luck, 1994). Fibronectin production and steroidogenesis represent two distinct states of granulosa cell differentiation, and it is proposed that fibronectin production is high in those tissues where extracellular matrix support is needed, like primordial follicles, atretic follicles and early corpus luteum (Luck, 1994). In bovine granulosa cells, fibronectin secretion is greater in confluent cultures of cells from large follicles (15 mm) and is lower in sparse cultures of cells from small follicles (4-5 mm Savion and Gospodarowicz, 1980).

The Theca Cell Compartment

Theca cells are the other cell type involved in follicular steroidogenesis. They are LH-responsive secretory cells which comprise the follicular envelope. The stem cell origin of theca cells is less clear than the origin of granulosa cells (Hirshfield, 1991). Theca cells appear to share a common ancestor with fibroblast and the stromal/connective tissue elements of the ovary (Gore-Langton and Armstrong, 1988). However, it has been suggested that granulosa and theca cells share a common ancestor (Hirshfield, 1991). Since the theca layer is not present in primary follicles but differentiates as follicles grow and mature, it is evident that theca cells arise continually throughout reproductive life. The aggregation and ultimate differentiation of the theca folliculi is assumed to occur under the influence of growth

factors emanating from the oocyte-granulosa complex, but there is no direct evidence to substantiate a casual relationship (Greenwald and Roy, 1994) The theca interna of sheep follicles, less than 3 mm in diameter, already consists of 8 to 12 layers of flattened cells and capillaries 20% of the cells have the ultrastructural features of steroidogenically active cells others are fibroblasts and the majority are undifferentiated cells In follicles 3-6 mm, approximately 40% of the theca interna possess tubular endoplasmic reticulum In all species the theca interna is separated from the granulosa cells by the basal lamina or basement membrane (Greenwald and Roy 1994)

The time of appearance of a well-differentiated theca interna relative to development of granulosa cells varies from species to species For example in sheep differentiation of the theca appears in follicles of 2-3 mm in diameter with maximal differentiation occurring in late estrus when numerous lipid droplets accumulate in theca interna cells Two cell populations emerge in the bovine theca interna at 3 to 4 days before ovulation One population consists of large epithelial cells with round nuclei increasing in area and another group of fibroblast-like cells in which the nuclear area does not increase In large (10 - 12 mm) porcine follicles, theca interna cells form a discrete layer, contain many more lipid droplets than granulosa cells and have a very high 3ß-hydroxysteroid dehydrogenase activity (Greenwald and Roy 1994)

The theca interna layer develops a vascular supply concomitant to its differentiation as a steroidogenic tissue (Greenwald and Roy, 1994) In the rat primordial follicles do not have an independent blood supply until multilaminar

granulosa and theca cell layers have formed. The vascular bed develops in the inner portion of the theca interna adjacent to the basal membrane and membrana granulosa. In turn, the theca interna vasculature is linked to an outer series of arterioles and venules in the theca externa layer (Greenwald and Roy, 1994). It is possible that follicular growth is angiogenesis-dependent. The increase in granulosal and thecal cells must be accompanied and/or preceded by an increase in new capillaries that grow toward and within the theca layer of growing follicles (Greenwald and Roy, 1994).

Two-Cell, Two-Gonadotropin Theory

In all mammalian species there are two types of cells involved in follicular steroidogenesis: LH-responsive secretory cells comprised of the theca interna cells of the follicular envelope and interstitial cells of the ovarian stroma, and FSH-responsive granulosa cells that acquire the ability to respond to LH during the later stage of follicular maturation. These two cell types fulfill distinct roles in the steroidogenic process by virtue of their different regulatory hormones and their dissimilar expression of steroidogenic enzymes (Gore-Langton and Armstrong, 1988). The studies of Falck (1959, cited by Richards, 1980) provided the first evidence that ovarian estrogen biosynthesis required the interplay of at least two ovarian cell types. He isolated granulosa, theca interstitial, and luteal cells and autotransplanted these cell types either alone or in combination to the eye chamber of ovariectomized female rats. Results from his experiments indicated that estrogen was produced only when theca or interstitial cells were cotransplanted with

granulosa cells or luteal cells or when intact follicles were autotransplanted (Richards, 1980)

The steroidogenic pathways present in granulosa and theca cells of developing follicles are responsible for the local as well as the systemic sex-steroid milieu. Three major classes of sex-steroids are produced by mammalian ovarian follicles: progestins, androgens, and estrogens, which enter the systemic circulation and exert important actions both within and outside the hypothalamo-pituitary gonadal axis (Urban and Veldhuis, 1992). Synergistic interactions between granulosa and theca cells have been proposed for some time as a mechanism by which ovarian follicles can produce the three classes of sex-steroids (Hsueh et al. 1984). This two-cell, two-gonadotropin theory of steroidogenesis can be summarized as follows:

Luteinizing hormone from the pituitary stimulates androgen production by theca cells (Fortune and Armstrong, 1977), which also appear to possess little or no ability to aromatize androgens to E_2 (Fortune and Armstrong, 1978). The locally produced androgens diffuse through the basement membrane to the granulosa cells. Follicle stimulating hormone stimulates aromatase enzyme activity in granulosa cells to allow conversion of androgens to estrogens (Fortune and Armstrong, 1977). Granulosa cells seem to be incapable of synthesizing androgens (Fortune and Armstrong, 1977) but produce progesterone *in vitro*. Granulosa cells appear to lack the 17α-hydroxylase, 17,20 desmolase enzyme necessary to continue the steroidogenic pathway to androgens (Fortune and Quirk, 1988). In

granulosa cells from immature hypophysectomized rats, aromatase enzyme is not present but can be induced by treatment with FSH (Dorrington et al., 1975).

Experiments with bovine theca and granulosa cells isolated from proestrous follicles have provided evidence that both theca and granulosa cells are necessary for follicular E_2 production in cattle (Fortune and Quirk, 1988). Androstenedione is the primary form of androgen secreted by bovine granulosa cells when cultured *in vitro* and stimulated by LH (Fortune, 1986). Luteinizing hormone and FSH increase production of P_4 by granulosa cells. Nevertheless, granulosa cells do not convert progestins to E_2 (Fortune, 1984). These observations led to the conclusion that the same two-cell, two-gonadotropin theory proposed for rats can be applied to the bovine follicle (Fortune and Quirk, 1988; Figure 2-1, model for regulation of E_2 production by bovine preovulatory follicles). Theca cells are stimulated by LH to synthesize androgens, but they are unable to convert androgens to E_2. Granulosa cells cannot synthesize androgens de novo, but they can convert exogenous androgens to E_2. It has been shown that granulosa cells can secrete E_2 in a long-term cell culture (Saumande, 1991; Rouillier et al., 1995; Diaz et al., 1997).

Fortune (1986) postulated that granulosa cells provide additional pregnenolone precursor (e.g., cholesterol) for thecal androgen production, which in turn enhances the ability of the granulosa cells to synthesize pregnenolone. This implies that cattle preferentially use the Δ^5 pathway to synthesize androgens (Fortune and Quirk, 1988). Estrogens secreted by granulosa cells act as a mitogen and, in conjunction with FSH, induces appearance of LH receptors on granulosa cells. In bovine follicles, E_2 exerts a positive feedback to stimulate its own

production and has a negative effect on P_4 secretion due to inhibition of the 3ß hydroxysteroid dehydrogenase in granulosa cells. Based on these observations Fortune and Quirk (1988) hypothesized that as the bovine follicle develops capability for E_2 production E_2 will inhibit conversion of pregnenolone to P_4 in both theca and granulosa cells. This increases the amount of pregnenolone available for conversion to androgens via the Δ^5 pathway in theca cells. Differential inhibitory effects of E_2 on P_4 versus androstenedione production could provide a mechanism by which E_2 initially exerts a positive feedback on its own production to increase androgen synthesis but eventually inhibits its own production by blocking thecal cell conversion of dehydroepiandrosterone to androstenedione through inhibition of 3ß-hydroxysteroid dehydrogenase (Fortune 1988).

Molecular Events Occurring in Theca and Granulosa Cells

Luteinizing hormone acts on theca cells via cAMP to regulate both C_{27} side change cleavage P-450 multienzyme complex (cholesterol 22-hydroxylase cholesterol 22α-hydroxylase and C_{20-22}-lyase) C_{21} side chain cleavage P-450 (17α-hydroxylase $C_{17\ 20}$-lyase) activation to increase androstenedione biosynthesis. In granulosa cells androstenedione (from thecal origin) is converted to testosterone by 17β-hydroxysteond dehydrogenase increasing the capacity of granulosa cells to synthesize pregnenolone (Fortune 1986). Estradiol then is synthesized from testosterone by the aromatase P-450 enzyme system. Estradiol in turn binds to E_2 receptors present in granulosa cells and may regulate gene expression (e.g. inhibin α- and $ß_B$-subunit mRNA Turner et al. 1989). However FSH must be

present to enhance intrafollicular E_2 effects (Richards 1980 Richards and Hedin 1988 Figure 2-1)

There is substantial evidence that the cAMP/protein kinase A (PKA) pathway is one of the most important regulatory mechanisms in theca cells Actions of LH on steroidogenesis of theca cells are mediated through the cAMP/PKA signal transduction pathway which stimulates the expression of steroidogenic enzyme genes required for androgen synthesis (P-450 side chain cleavage 3ß-HSD and P-450 17α-hydroxylase 17 20-lyase) Once theca cells have expressed the steroidogenic enzymes activation of the cAMP/PKA pathway also stimulates the rate-limiting step in steroid hormone biosynthesis which is the transfer of cholesterol across mitochondrial membranes (Magoffin and Erickson 1994) However a number of observations has indicated that the activity and amount of of the P-450 side chain cleavage enzyme are unaffected by trophic hormone stimulation (Stocco 1997) Recently it has been thought that the rate-limiting step in steroidogenesis is the de novo synthesis of a protein whose function is to facilitate the transfer of the substrate cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane and the P-450 side chain cleavage enzyme. The synthesis of this protein is dependent upon a functional cAMP-dependent protein kinase A signaling pathway that is linked to the steroidogenic potential of the cell

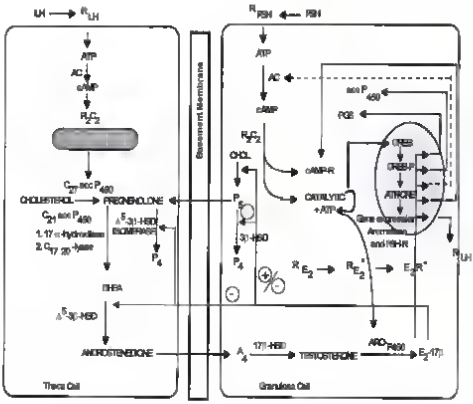

Figure 2.1 Schematic representation of the molecular events coordinating estradiol, FSH, and LH action in theca and granulosa cells of preovulatory follicles (Adapted from Richards and Hedin 1988; Gore-Langton and Armstrong, 1988; Fortuna and Quirk, 1988)

The protein was named the Steroidogenic Acute Regulatory (StAR) protein (Stocco 1997). Interaction of StAR with the mitochondria may cause the formation of a protein complex consisting of P-450 side chain cleavage and 3β-HSD enzymes required for the first two steps in steroidogenesis (Stocco 1997). It appears that cAMP in theca cells increases the content of the regulatory (R) subunit of cAMP-dependent protein kinase type II (RII_{51}). P-450 side chain cleavage enzyme and 17α-hydroxylase 17, 20-lyase

receptors have little or no effect on proliferation of granulosa cells unless FSH acts via the FSH receptor to increase intracellular concentrations of cAMP (Richards, 1980) On the other hand E_2 is required for FSH to induce LH receptors in granulosa cells The effects of E_2 on granulosa cell function are mediated via the translocation of the cytosol E_2-receptor complex to the nucleus Binding of the E_2-receptor complex to nuclear acceptor sites results in altered genomic expression synthesis of new messenger RNA and a change in any one of a number of the components of the FSH receptor system It is also possible that E_2 enhances the ability of FSH to stimulate the adenylate cyclase system Estradiol may increase the content of a specific cAMP binding protein that is distinct from the regulatory subunit of the protein kinase or is the regulatory subunit of a specific protein kinase Estradiol could also induce the synthesis of a specific protein that when phosphorylated in the presence of active kinase might enter the nucleus to affect gene transcription (Richards 1980)

Gonadotropic hormone control of granulosa cell steroidogenesis occurs primarily by way of cAMP-dependent pathways but evidence exists that additional intracellular regulatory signals can modify this process Two such second-messenger systems involve intracellular hydrolysis of phosphatidylinositol to produce inositol trisphosphate (IP_3) and diacylglycerol. Intracellular Ca^{++} is increased in response to IP_3 which in turn affects activity of protein kinase C and activation of the calcium-calmodulin system (Urban and Veldhuis 1992)

Hormonal Control of Ovarian Follicular Dynamics

During each estrous cycle, bovine ovaries synthesize and secrete E_2 17ß and P_4 which coordinate function of the female reproductive system. Each estrous cycle is comprised of follicular and luteal phases. The follicular phase is characterized by development of the preovulatory follicle and its secretion of E_2 whereas the luteal phase is characterized by secretion of P_4 by the corpus luteum which is formed after ovulation of the preovulatory follicle. At the end of the luteal phase the corpus luteum regresses by the action of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and final development of the next preovulatory follicle occurs. Gonadotropin secretion plays a central role in control of the estrous cycle. The developing preovulatory follicle produces a critical level of E_2 that stimulates the hypothalamus to increase the frequency and amplitude of gonadotropin-releasing hormone pulses(GnRH). In cattle this is inferred based on an increased frequency and amplitude of LH pulses. Increased LH pulses amplify E_2 secretion, complete follicular development, induce estrous behaviour and trigger the preovulatory surge of LH. Ovulation occurs ~ 30 h after the preovulatory LH surge (Chenault et al. 1975; Robinson and Shelton 1991; Driancourt et al. 1993a).

Steroid Hormones

Steroid hormones such as progestins, androgens and estrogens are produced by the ovarian follicle during its development. Each wave of development of a dominant follicle goes through a selection, dominance and atresia or ovulation

phase. During the selection phase E_2 production by each ovary is similar (Ireland and Roche 1987). At the end of this phase one follicle becomes larger than all other follicles and is responsible for most of the E_2 production and contributes to a transient increase in concentration of E_2 in the peripheral circulation. If luteal regression occurs coincident with the dominance phase in development of a dominant follicle then the follicle ovulates. If luteal regression does not occur during a dominance phase, the dominant follicle undergoes atresia and a new selection phase begins (Ireland and Roche 1987). In cows the preovulatory surge of LH is induced by increasing concentrations of E_2 after demise of the corpus luteum (Chenault et al. 1975, 1976; Kesner et al. 1981). Estradiol initiates the preovulatory surge of LH by acting on the hypothalamus to increase secretion of GnRH at the pituitary level and the sensitivity of the gonadotropes to GnRH. A preovulatory surge of FSH occurs concomitantly with the surge of LH (Walters and Schallenberger 1984). The preovulatory follicle is the source of this increase in E_2. Plasma E_2 concentrations are high before the LH surge and decline during the surge. Follicular fluid concentrations of androgens and progestins are much lower than E_2 before the LH surge. Following the surge androgens decline in follicular fluid whereas P_4 increases. In contrast atretic follicles differ from healthy follicles in that they have much lower concentrations of E_2 than healthy follicles (Fortune and Hansel 1985; Badinga et al. 1992; de la Sota 1995).

Demise of the first dominant follicle at midcycle is due to the negative feedback effect of progesterone from the corpus luteum on LH secretion (Savio et al. 1993a). In the absence of a normal corpus luteum and in an environment with

low progestin the first dominant follicle continues to grow and suppresses growth of other follicles for more than 20 days (Savio et al 1993a) This sustained growth and functional dominance is due to increased LH pulse frequency The presence of high levels of P_4 during the luteal phase assures that the period of functional dominance of any follicle is limited due to induction of a low LH pulse frequency that is insufficient to maintain follicular function and leads to atresia. In contrast, maintenance of P_4 concentrations between 1 and 2 ng/ml prolongs both morphological and functional follicular dominance (Sirois and Fortune 1990 Savio et al 1993b) In cases where the estrous cycle is lengthened by maintaining normal luteal concentrations of P_4 either artificially or naturally as during pregnancy (Ginther et al 1989c) continuous follicular waves occur However occurrence of follicle waves in pregnancy is more prevalent on the ovary contralateral to the uterine horn bearing the conceptus

Gonadotropic Hormones

Regulation of steroid production by bovine theca and granulosa cells seems to depend on gonadotropins and on paracrine regulation by ovarian hormones Theca cells have LH but not FSH receptors (Ireland and Roche 1983) Binding of LH to theca cells increases as the preovulatory follicle develops In contrast, granulosa cells can bind both LH and FSH As the preovulatory follicle develops its granulosa cells increase their specific binding of LH but exhibit decreased binding of FSH (Ireland and Roche 1983) Both LH and FSH are essential for follicular development and steroidogenesis in vivo (Fortune et al 1988)

Follicle stimulating hormone is the key hormone stimulating emergence of follicular waves and declines in FSH are associated with selection of a dominant follicle which becomes dependent on LH for its final fate (Roche, 1996). Adams et al. (1992b) concluded that there is a temporal sequence between surges of FSH and subsequent emergence of follicular waves. Time of follicle selection coincides with the first decrease in FSH secretion. Turzillo and Fortune (1990) reported that the secondary FSH surge (e.g. at 24 h after the FSH preovulatory surge) is important for the initiation of follicular development early in the bovine estrous cycle. Adams et al. (1992b) and Sunderland et al. (1994) reported that there is a cyclic pattern of FSH secretion during the estrous cycle of cattle with enhanced FSH secretion responsible for emergence of each follicle wave. Circulating concentrations of FSH decrease once selection is initiated as indicated by the presence of one estrogen-active follicle. This suggests that the dominant follicle secretes some inhibitory substance(s) to decrease FSH. The dominant follicle continues to grow demonstrating that large amounts of FSH are not necessary to sustain follicular dominance.

Terminal stages of antral follicular development and follicular hierarchy in monovular species may be regulated at the ovarian level by the presence of an active dominant follicle. Turnover of dominant follicles which permits development of new follicular waves appears to be determined by the inability of the dominant follicle to continue growing and suppressing growth of other follicles. There are physiological conditions which support the concept that LH exerts a regulatory effect on growth and dominance of dominant non-ovulatory and ovulatory follicles in cattle.

Such situations include the early postpartum period in dairy and beef cows in which development of follicles ≤ 9 mm occurs (Savio et al 1990b) In beef cows during the early postpartum period two to three non-ovulatory follicles develop before the first postpartum ovulatory follicle develops (Murphy et al 1990) During these two situations LH concentrations are at basal levels Also growth and ovulation of follicles in prepubertal heifers can be achieved by repeated injections of LH (Tortonese et al 1990)

In summary turnover of ovarian follicles during the estrous cycle in cattle is regulated by the concentration of P_4 in plasma acting via a negative feedback effect on LH secretion Low frequency of LH pulses during the luteal phase is not sufficient to maintain continued growth and function of the dominant follicle It is probable that a low androgen secretion by theca cells limits subsequent function of granulosa cells Functional granulosa cells are required for terminal follicular development and sustained dominance of the follicle. When granulosa cell functional is compromised the dominant follicle no longer suppresses growth of other follicles and this leads to recruitment of a new follicular wave (Savio et al 1993a) Enhanced FSH secretion is also responsible for development of the next follicular wave Adams et al (1992b) showed that a surge in circulating FSH occurred during the plateau phase in maximal diameter of an anovulatory dominant follicle and that the FSH surge was related to detectable emergence (4 or 5 mm follicles) of a follicular wave

Other Factors Involved in Control of Ovarian Follicular Dynamics

Growth and development of a tissue requires the local production and integrated actions of specific growth factors. These growth factors mediate critical cell-cell interactions that control cell proliferation and organ development. Gonadal development also requires growth factor-mediated cell-cell interactions as a general mechanism to control cellular proliferation. Ovarian physiology requires rapid and continuous growth regulation associated with the process of folliculogenesis (Skinner, 1992). There is increasing evidence that growth factors modulate folliculogenesis but their precise role in the processes of follicular growth, differentiation and atresia is still unknown (Monget and Monniaux, 1995). Growth factor-mediated interactions between theca cells, granulosa cells, and the oocyte are required for the maintenance of ovarian function and the process of oogenesis. In most large animals, follicle size increases from millimeter to centimeter (e.g., cow, mare). Granulosa and theca cells are responsible for this follicular expansion. In addition to cell proliferation required during follicle development, follicles of various stages of development become atretic and cell growth is arrested. Regulation of cell proliferation in the follicle requires stimulatory and inhibitory growth factors (Skinner, 1992).

Ovarian follicular dynamics are controlled not only by the interaction of endocrine signals (steroid and gonadotropic hormones) but by locally produced ovarian peptide hormones and growth factors (Monget and Monniaux, 1995). The responses of the two major follicular cell types, granulosa and theca cells, to

gonadotropins is regulated by the local production of growth factors and peptides Ovarian follicles are known to produce a range of locally acting peptide/protein growth factors that can interact directly with the same cell type from which they are produced in an autocrine manner or with other cell types via a paracrine action to attenuate or stimulate the cellular response to gonadotropins Also these factors could act in a juxtacrine fashion activating receptors on adjacent cells or stimulate the cell in which the factor is secreted without prior secretion of the factor from the cell (intracrine action Armstrong and Webb 1997)

Inhibins activin IGF I IGF-II IGF binding proteins TGF-α EGF and TGF-ß and other facors have direct and indirect effects on granulosa and theca cells that can modulate follicular development and steroidogeneses Inhibins have both autocrine and paracrine effects They increase LH-induced androgen synthesis in theca cells (Hillier et al 1991b Wrathall and Knight, 1995) and inhibin production is stimulated by steroids and FSH (Wrathall and Knight 1993) This is indicative of a local feedback loop within individual follicles involving a sequential change of inhibins activins and their binding proteins which determine the different fates of the selected and unselected follicles that develop in the same systemic environment of gonadotropins and growth factors (Roche 1996) There is evidence mainly from studies *in vitro* that the inhibin-related peptides have actions on all the functional stages of folliculogenesis including ovulation (Findlay 1993)

Inhibin

The term "inhibin" was proposed originally by McCullagh in 1932 to denote the activity of an aqueous extract of the testis that had the capacity to suppress formation of castration cells (cells that appear in the pituitary following damage of the seminiferous tubules) in the anterior pituitary gland. This was before the time when gonadotropins, FSH and LH, were defined as separate hormones and before testosterone was isolated as a pure substance. The term inhibin was used to designate a non-steroidal gonadal product with the capacity to specifically suppress FSH secretion. However, isolation of this molecule did not occur until 53 years after the original postulate (de Kretser and Robertson, 1989). Inhibin is a slow acting but powerful inhibitor of pituitary FSH biosynthesis and secretion by the anterior pituitary gland (Gaddy-Kurten et al., 1995).

Inhibin is a heterodimeric glycoprotein composed of an α subunit (relative molecular mass, M_r = 18 kDa) linked by a disulfide bridge to one of two highly homologous ß-subunits (approximate M = 14 kDa) to form either inhibin A (α-ßA) or inhibin B (α-ßB; Findlay et al., 1993; Rose and Gaines-Das, 1996; Halvorson and DeCherney, 1996). By sequence analysis of cDNA clones, it has been shown that the three subunits (α, $ß_A$ and $ß_B$) are coded by different genes, and the mature structures of the α, ßA- and ßB subunits are present at the C terminus of much larger precursor protein. The α subunit precursor is composed of four segments: a signal peptide, prosequence (Pro), N-terminal (αN) and C terminal (αC) peptides in addition to the α component. The 31-kDa inhibin has been postulated as the

mature form of inhibin in the circulation (Findlay et al 1993). Robertson et al (1985, 1986) using a fractionation procedure reported the identification of a range of bioactive and immunoactive forms of inhibin in human plasma. These forms have molecular masses of 28 to 126 kDa attributed to differences in glycosylation of the α subunit and differential processing of the α and ß subunits. The inhibin ß sequences also exhibit close homology with the TGF ß superfamily of structurally related proteins with wide-spread biologic activities (Massague 1987). Granulosa cells are the site of expression of mRNA encoding the different inhibin subunits in bovine antral follicles (Torney et al 1989, Findlay et al 1993).

Inhibin has a negative feedback effect on secretion of FSH (De Jong, 1988, Glencross et al 1994). Inhibin is likely a chemical signal of the number of growing follicles in the ovary (Taya et al. 1996) and through its systemic action inhibin probably has an important role in determining species-specific ovulation rates (Taya et al 1996, e.g. ovulation of only one follicle in cattle, Scanlon et al 1993). The initial isolation of inhibin was achieved from bovine follicular fluid (bFF) as a 58 kDa glycoprotein consisting of two disulphide-linked subunits of apparent molecular masses of 43 kDa and 15 kDa. The larger subunit has been termed α and the smaller as the ß-subunit (Robertson et al 1985). Ling et al (1985) isolated two forms of inhibin, termed inhibin A and inhibin B, which differs by the NH_2 terminal amino acid sequences of their ß-subunits which are now termed $ß_A$ and $ß_B$. More recently, Good et al (1995) isolated nine different biologically and immunologically active molecular variants of inhibin (29, 34, 49, 53, 58, 77, 99, 110, and > 160 kDa) from bovine follicular fluid. Quantitative immunoblot analysis has shown alterations

in the nine different molecular variants in bovine follicular fluid associated with functional status of dominant follicles. The predominant inhibin forms are free α subunits and large (> 34 kDa) inhibin forms rather than the fully processed inhibin form (Ireland et al. 1994). According to Good et al. (1995) each form of inhibin except the 29-kDa form inhibited basal FSH secretion and enhanced GnRH-induced LH secretion in pituitary bioassays. The dimeric forms of inhibin retained biological activity after isolation from bovine follicular fluid.

Granulosa cells of the follicle and luteal cells in primates produce inhibin and its production can be regulated via endocrine (FSH, LH), paracrine (EGF, TGF-α, interferon-γ, androstenedione) and autocrine (IGF-I, TGF-β, activin, follistatin) controls (Findlay, 1993). Follicle stimulating hormone, LH (at low doses), IGF-I, TGF-β, and activin can stimulate inhibin production by granulosa cells. Epidermal growth factor, TGF-α, follistatin, interferon-γ, and high doses of LH negatively regulate inhibin production in the presence of FSH and other agents that increase cAMP concentrations (Findaly et al., 1993). Secretion of inhibin by rat, bovine (Henderson and Franchimont, 1983), human, and nonhuman primate granulosa cells is regulated by gonadotropins and sex steroids in vitro (Hillier and Tumer, 1991). The expression of the inhibin subunit genes is regulated developmentally and inducible by FSH in the rat (Woodruff et al., 1987, 1987). In cultured rat granulosa cells, expression of α- and β_B-subunit mRNA is enhanced by E_2. This suggests that there is a mechanism whereby locally produced estrogen could influence relative rates of inhibin and activin synthesis by granulosa cells during follicular development in vivo (Hillier and Tumer, 1991).

Ireland and Ireland (1994) found a high correlation of α and $ß_A$ mRNA with intrafollicular changes in E_2, P_4, E_2-P_4 ratio and total inhibin immunoactivity during growth of nonovulatory follicles. Ireland and Ireland (1994) reported that amounts of follicular inhibin α and $ß_A$ mRNAs increased coincident with increases in size of nonovulatory follicles and expression was reduced markedly in atretic nonovulatory follicles compared with estrogen-active follicles. It was also shown that amounts of $ß_B$ mRNA remained unchanged during follicular development.

De le Sota (1995) reported that there are two very distinct patterns of change in the amounts of immunoblot bovine inhibin in follicular fluid of dominant and subordinate follicles on day 5, 9 and 12 of the estrous cycle. Absolute amounts of four forms (>160, 160-110, 77 and 49 kDa) were high in dominant follicles of day 5 and 9, but lower in atretic dominant follicle of day 12. Conversely, absolute amounts of the 31 kDa form of bovine inhibin in follicular fluid increased with atresia of the dominant follicle. In heifers, intrafollicular concentrations of inhibin decrease during growth of dominant ovulatory follicles, but increase during growth of dominant non-ovulatory follicle (Martin et al., 1991). Thus, inhibins may have an important role in regulation of growth, differentiation and ovulatory quota of dominant follicles during the estrous cycle in heifers. It has been shown (Scanlon et al., 1993) that immunization of beef heifers with α (1-26 Gly-Tyr) subunit of 32 kDa bovine inhibin conjugated to human serum albumin increased ovulation rate in 32% of estrous cycles of heifers. Five injections of steroid-free bovine follicular fluid (which contained inhibin), given every 12 h beginning 12 h after the onset of estrus, suppressed completely the secondary surge of FSH without affecting LH

concentrations. This treatment delayed the appearance of the first wave of follicular development (Turzillo and Fortuna, 1990).

Sunderland et al. (1996) demonstrated that the gonadotropic surge causes changes in levels or proportions of certain forms of inhibins. There was no change of inhibin forms during the period of most active E_2 synthesis (preovulatory phase). After the gonadotropic surge, the 110-kDa form decreased while the 29-kDa inhibin form and total inhibin immunoactivity increased. In addition to gonadotropic regulation of inhibin production, paracrine and autocrine factors are also involved. First, steroids have the ability to change the secretion of inhibin in vitro. In rat granulosa cell cultures, FSH induced immunoreactive inhibin production was increased by androstenedione and E_2, whereas in the absence of FSH neither steroid was effective (Findlay et al., 1993). Various growth factors have also been demonstrated to influence inhibin production. Both IGF-I and TGF-ß increase basal and FSH-induced inhibin production by rat granulosa cells. In contrast, TGF-ß inhibited basal and FSH-stimulated inhibin production by pig granulosa cells (Michel et al., 1991). Epidermal growth factor appears to be a negative regulator of both inhibin protein (Michel et al., 1991) and α and ßA subunit mRNA (LaPolt et al., 1990). Interferon-γ is also a negative regulator of inhibin production by cultured immature rat granulosa cells, but only in the presence of FSH or other agents that increase cAMP levels (Xiao and Findlay, 1990).

In sheep, Campbell et al. (1996) reported that granulosa cells from large follicles secreted E_2 in response to FSH and secreted inhibin and P_4 regardless of the presence or absence of FSH. Insulin and LR3 IGF-I (human recombinant Long

R3 IGF-I) had a significant interaction in the stimulation of hormone production by granulosa cells from small and large follicles. On the other hand, insulin alone stimulated E_2 and inhibin secretion.

A receptor with specific affinity for inhibin has not been demonstrated. However, inhibin has been shown to bind to activin type II receptors although with lower affinity than activin. Therefore, rather than acting through a unique receptor, inhibin may exert its effect through competition with activin for activin receptor sites (Gaddy-Kurten et al., 1995; Halvorson and DeCherney, 1996).

Activin

Inhibin and activin are closely related peptides. Activin is composed of homodimers or heterodimers of the same ß-subunits linked by interchain disulfide bonds resulting in activin-A (ßA-ßA), activin-AB (ßA-ßB) or activin-B (ßB-ßB; Halvorson and DeCherney, 1996). The molecular weight of activin is 24 to 28 kDa (Findlay et al., 1993). The ß-subunit mRNAs are translated into pre-pro-ß forms which are proteolytically processed to the mature ß-subunits. Activin A has been isolated from bovine follicular fluid, but activin B has not been isolated from mammalian sources so far (Findlay et al., 1993). Robertson et al. (1992) showed that the ßA subunit monomer is present in bovine follicular fluid at a level 25% to 50% that of the ßA subunit dimer (activin A), and its effect on in vitro responses are similar to those of the dimer. However, the monomer is less immunologiosly and biologically active (15% to 45%) than the dimer. It is unclear if dimerization of the monomer is a necessary prerequisite for biologic activity.

Activin is a product of the granulosa cells as determined from expression of the ß subunit mRNA in these cells and the isolation of activin from follicular fluid (Findlay 1993 Findlay et al 1993) Activin has the ability to stimulate FSH production and decrease inhibin biopotency (Robertson et al 1989) The activin/inhibin ßB subunit is expressed within rat gonadotropic cells is locally secreted as ßBßB activin and may function as an autocrine modulator of basal FSH secretion and expression Inhibin and activin are part of a larger superfamily that includes transforming growth factor (TGF)-ß müllerian inhibiting substance the decapentaplegic gene complex of Drosophila the bone morphogenic proteins and the vegetal growth factor gene of Xenopus (Mather et al 1992)

It is not known why different isoforms of inhibin and activin exist It has been proposed that they may have different potencies or serve divergent functions Also regulatory factors that determine the preferential formation of α-ß (inhibin) versus ß-ß (activin) dimers in cells that produce both subunits are poorly understood Differential expression of the two subunits probably serves as one regulatory mechanism in which an excess of α-subunit shifts production toward inhibin (Li et al 1995 Halvorson and DeCherney 1995) Activin is recognized as a regulator of a variety of actions including neuronal differentiation neuronal lifespan and mesoderm induction in Xenopus (Mather et al 1992)

Activin affects the differentiation of granulosa cells in an autocrine manner and its action depends on the stage of differentiation of the cells It promotes differentiation of cells during the preantral and early antral stages of folliculogenesis and prevents premature luteinization of cells from the later stages of antral

development The coordinated effects lead to a promotion and maintenance of folliculogenesis (Findlay 1993) Data from rat and bovine granulosa cell models support the hypothesis that activin regulates granulosa cell differentiation in an autocrine fashion and this action of activin is related to the stage of granulosa cell differentiation (Findlay 1993)

Activin is not only a mitogen for granulosa cells but seems to play an important role in granulosa cell differentiation and ovarian follicular morphogenesis Hulshof et al (1997) reported that activin-A and activin receptor were co-localized in oocytes and granulosa cells of preantral follicles suggesting an autocrine action of activin on preantral follicles Activin stimulated both an increase in diameter of preantral follicles and proliferation of the granulosa cells Li et al (1995) showed that activin induced reaggregation of granulosa cells into a follicle-like structure Activin (≥ 100 ng/ml) enhanced FSH-induced aromatase activity (Hutchinson et al 1987) LH binding sites P_4 production FSH-induced inhibin levels and both α and ß subunit mRNA (Xiao et al 1990) The mechanisms of activin action are likely to involve stimulation of FSH receptor formation and cAMP production (Findlay et al 1993) In addition activin increased the responsiveness of granulosa cells to both gonadotropins (Xiao and Findlay 1991) These in vitro observations are consistent with activin acting as an autocrine regulator to promote folliculogenesis during the preantral or early antral stages Mainly activin induces FSH receptors as a mechanism whereby preantral follicles may become responsive to FSH (Findlay et al. 1993) This proposition assumes that the granulosa cells either constitutively express or acquire receptors for activin prior to this important step in differentiation

(Findlay 1993) It is proposed that activin produced by granulosa cells of preantral follicles lacking FSH receptors acts on these same cells to induce FSH receptors The FSH then acts on the cells to stimulate production of more activin and the activin receptor and FSH and activin together further enhance FSH receptor number in partially differentiated cells FSH and activin also induce follistatin production, which modulates the action of activin and may have an effect on the granulosa cell independent of its activin binding properties This model also implies that some differentiating actions of FSH on granulosa cells may be mediated by activin which would explain why follistatin inhibits FSH-induced differentiation of the cells in vitro (Findlay 1993)

Activin action on differentiated granulosa cells is consistent with a role in preventing prematura luteinization (Findlay 1993) In bovine granulosa cell cultures recombinant human activin A caused a time- and dose-dependent inhibitory effect on LH-induced production of P_4 and oxytocin (Shukovski et al. 1991) Bovine activin A prevented the spontaneous luteinization of fully differentiated bovine granulosa cells in vitro (Shukovski and Findlay 1990) Activin is also a potential autocrine modulator of aromatization in granulosa cells (Hillier 1991) It has been shown that activin is an steroidogenic agent (Woodruff et al 1990) Exposure of rat and human theca cells to activin results in a dose-dependent reduction in LH-induced androstenedione production (Hsueh et al. 1987) This would constitute a paracrine action of activin in the ovarian follicle (granulosa-thecal cells regulation) and could have important implications in the establishment of dominance in follicles (Findlay 1993) These results suggest that

activin plays an important role in regulating the functional integrity and development of granulosa cells in the ovarian follicle and these effects may be mediated directly through activin receptors on granulosa cells

Activin receptors can be divided into two classes: type I (actRI) and type II (actRII) ActRI binds activin with high affinity however binding only occurs in the presence of the signaling peptide actRII Conversely actRII activity depends on the formation of a noncovalent heteromeric complex with actRI (Mathews 1994) This complex functions as a serine-threonine kinase (Halvorson and DeCherney 1996) Multiple isoforms have been detected within the activin type I and type II receptor classes Each isoform demonstrates distinct ligand-binding affinities and cytoplasmic domain structure This observation raises the possibility that dose- and cell-specific responses could be attained through the expression of various combinations of activin receptor isoforms and number (Halvorson and DeCherney 1996) Type I receptors are expressed and reach the cell membrane even in the absence of the type II receptors Type I receptors do not bind ligand without the coexpression of a type II receptor Both classes are required for any response (Gaddy Kurten et al 1995)

Activin and inhibin share a common ß subunit, and both bind to α_2-macroglobulins and to follistatin, which is a specific activin ß subunit-binding protein (Gaddy-Kurten et al., 1995) Although the physiological relevance of the inhibin-α_2-macroglobulin and activin-α_2-macroglobulin interaction is unknown both complexes are biologically active to modulate FSH secretion Although most activin appears to be complexed with α_2-macroglobulin complex formation probably does not affect

activin function. The α_2-macroglobulin presumably binds activin with lower affinity than follistatin or cell surface receptors which could account for the lack of effect on activin bioactivity alternatively activin may be able to interact with its receptor while complexed to α_2-macroglobulins. The physiological function of this complex formation is not known but α_2-macroglobulins may serve roles in storage delivery and clearance of activin (Mathews 1994). In contrast activin-follistatin complex is biologically inactive and it is likely that this binding protein plays an important role in limiting exposure of cells to activin (Gaddy Kurten et al. 1995).

Follistatin

Follistatin is a single peptide chain (molecular weight 32 000 – 35 000 Daltons) that was first isolated from porcine follicular fluid and is distinct from inhibin and activin. This cysteine-rich protein can inhibit the release but not the synthesis of FSH and has no effect on LH in cultures of pituitary cells (Tonetta and diZerega 1989). Follistatin is structurally related to the activin-inhibin subunits. The primary structure of follistatin across species is highly conserved (> 87%). Follistatin is coded by a single gene organized into three homologous domains each with > 50% homology. Alternative mRNA splicing results in two mature mRNA forms the longer encoding FS-315 and the shorter encoding FS-288. The existence of two isoforms of follistatin provides cells with a mechanism to control follistatin activity through alterations in post-transcriptional processing (Halvorson and DeCherney 1996). Follistatin is expressed in a number of different tissues, but

a major site of production is the ovarian granulosa cells under FSH regulation (Findlay et al 1993)

It has been demonstrated that follistatin mRNA and protein expression change in association with folliculogenesis and atresia (Findlay et al., 1993) Follistatin mRNA is first detected in granulosa cells of secondary follicles and then becomes more abundant as the follicle forms an antrum, with uniform expression in the whole granulosa cell layer The strongest signal is found in preovulatory follicles and newly formed corpora lutea Follistatin protein is confined to healthy dominant preovulatory follicles and a subpopulation of tertiary follicles in animals entering estrus (Shukovski et al 1992) Atretic follicles show no immunoreactive follistatin in granulosa cells The bovine follistatin mRNA signal increases as folliculogenesis progresses with the strongest signal being observed in preovulatory bovine follicles (Shukovski et al 1992)

Findlay (1993) and Findlay et al (1993) hypothesized that follistatin modulates granulosa cell function in an autocrine fashion and that follistatin action is through binding and neutralization of activin (Mathews 1994) Thus, follistatin is likely to favor the process of follicular luteinization or atresia However follistatin may also have direct actions on granulosa cells independent of its activin-binding activity Xiao et al (1990) reported that follistatin enhanced the stimulatory action of forskolin on P_4 production by rat granulosa cells but did not influence the effect of forskolin on either aromatase activity or inhibin production In contrast, activin enhanced all three responses stimulated by forskolin The binding of activin to follistatin is through two binding sites present in activin-A (β_A-β_A) whereas inhibin-A

(α-β_A) has only one binding site for follistatin. Shimoneka et al. (1991) hypothesized that follistatin binds activin and inhibin through the common ß subunit.

There is strong evidence for a short loop feedback system involving activin and follistatin in the ovarian follicle. Follicle-stimulating hormone can stimulate production of both activin and follistatin. Follistatin can neutralize activin action and possibly some actions of FSH that might be mediated by activin (Findlay et al. 1993). The production of the activin-binding protein follistatin in the same tissue may further limit the bioavailability of activin and inhibit activin-induced cell growth and formation of follicles, as reported by Li et al. (1995). Alternatively because the affinity of activin for follistatin is comparable to that of activin for its receptors, follistatin could directly compete with activin receptors of lower affinity for activin. However, higher affinity activin receptors could still bind ligand and elicit signal transduction (Mathews, 1994).

Transforming Growth Factor ß

Transforming growth factor-ß is a family of multifunctional growth factors originally named for their ability to induce normal rat kidney fibroblasts to grow in soft agar in the presence of epidermal growth factor (EGF, Mulheron and Schomberg, 1993). The TGF-ß superfamily is made up of a number of proteins with the potential to act as intraovarian regulators of ovarian function (Armstrong and Webb, 1997). Also, müllerian inhibiting substance is included among the members of the TGF ß family (Mulheron and Schomberg, 1993). Transforming growth factors-ß are ubiquitous peptides and have been shown to affect nearly every cell

in the body. There are five TGF-ß subtypes, three of which (TGF-ß1, TGF-ß2 and TGF-ß3) are expressed by cells of the mammalian ovary (Hernandez et al. 1990; Mulheron and Schomberg, 1990). They are products of separate genes, but few cellular responses to TGF-ß can be ascribed solely to one isoform. Transforming growth factor-ß is synthesized as an inactive precursor protein that must be cleaved for the 25-kDa homodimer to elicit its effects.

The precise mechanism of TGF-ß activation in vivo is unknown, but is thought to occur via induction of proteases such as plasmin or perhaps by processing in an acidic environment. In vitro TGF ß can be activated by exposure to heat, detergent, or acid. Once activated, TGF-ß is thought to affect target cells through TGF-ß-specific membrane-bound receptors, but the precise cellular mechanisms of TGF-ß actions have not been elucidated (Mulheron and Schomberg, 1993). The most likely mediators of TGF-ß signal transduction are three cell surface proteins, which were identified based on their ability to bind and be chemically cross-linked to radiolabeled TGF-ß (Massague and Like, 1985). These three molecules have been designated type I, II, and III receptors and have apparent molecular weights of 55, 80, and 280 kDa, respectively. Data from a variety of studies indicate that receptors types I and II are responsible primarily for signal transduction. The type III receptor, a membrane-anchored proteoglycan, also called betaglycan, that may help modulate cellular access to TGF-ß by sequestering it near the cell membrane. (Mulheron and Schomberg, 1993). The type II receptor has a functional cytoplasmic serine/threonine kinase domain, which suggests that serine/threonine phosphorylation may be an important component in the intracellular

signal transduction of TGF ß The type II receptor is structurally related to the activin receptor Regulation of TGF ß activity is controlled by the activity of proteases that release mature TGF-ß from the extracellular matrix-associated latent complex The conversion of plasminogen to plasmin within the follicle and the presence of plasminogen activator inhibitors would be expected to be critical in the control of TGF ß activity during folliculogenesis This mechanism would be expected to influence development of dominance since TGF-ßs act synergistically with gonadotropins to control the differentiation of follicular cells Specific extracellular proteases play a central role in the regulation of TGF ß bioactivity during follicle growth and development of dominance (Armstrong and Webb 1997)

Given that TGF-ß is a multifunctional growth factor it can act as both a growth stimulator and an inhibitor depending on cell type and culture conditions Transforming growth factor-ß is primarily an inhibitor of follicle growth and therefore facilitates follicle cell differentiation by modulating granulosa cell expression of functional gonadotropin receptors (Mulheron and Schomberg 1993) Ovarian theca cells have been shown to express and produce TGF ß (Skinner et al 1987a) Transforming growth factor-ß has been shown to stimulate a number of granulosa cell functions, including FSH induction of LH receptors EGF actions FSH induction of aromatase activity production of IGF-I and inhibin Transforming growth factor-ß also can influence theca cell function and steroidogenesis and oocyte maturation (Skinner 1992) Transforming growth factor ß inhibits growth of bovine and porcine granulosa cells (Skinner et al 1987a) and theca cells (Roberts and Skinner 1991) induced by TGF-α/EGF The influence of TGF-ß on cell function

may be mediated indirectly through inhibition of cellular proliferation. Growth inhibition may be important to prevent premature cell growth of the preantral follicle, arrest cell growth during atresia, and control cell growth during follicle cell expansion (Skinner 1992). Overall, these data indicate that TGF-ßs are local regulators of ovarian function (Armstrong and Webb 1997). In cows, TGF-ßs inhibit granulosa and theca cell proliferation while enhancing gonadotropin-stimulated steroidogenesis (Roberts and Skinner 1991).

Epidermal Growth Factor/Transforming Growth Factor-α

Other growth factors known to affect ovarian function include TGF-α, a structural analogue of EGF. Transforming growth factor-α is expressed in bovine theca cells and has been localized in the thecal cell (Skinner and Coffey 1988) layer during follicular growth. However, there is a decrease in immunoreactive TGF-α in preovulatory follicles that is correlated with a decrease in mitotic activity of granulosa cells (Lobb and Dorrington 1992). Epidermal growth factor and TGF-α are products of separate genes; however, they are structurally similar and both elicit their effects through the EGF receptor (Mulheron and Schomberg 1993). Epidermal growth factor and TGF-α are believed to be produced by ovarian theca cells (Skinner et al. 1987b).

Epidermal growth factor is a small (53 amino acid, 6043 molecular weight) single-chain polypeptide containing six half-cysteine residues that form three intrachain disulfide bonds. It is synthesized as a large molecular weight precursor that can be proteolitically cleaved to generate the biologically active form. Within

species, two to six EGF isoforms have been elucidated that have variable degrees of receptor binding and bioactivity (Mulheron and Schomberg 1993).

Transforming growth factor-α is synthesized as a glycosylated precursor protein of 159 or 160 amino acids with the mature 50-amino acid form being generated by proteolytic cleavage. The precursor form contains a signal peptide sequence and a hydrophobic transmembrane domain suggesting that the precursor form of TGF-α is an integral membrane glycoprotein (Mulheron and Schomberg, 1993). Membrane-anchored TGF-α binds to EGF receptors on adjacent cells and elicits a response. Such a mechanism would allow for activation of adjacent cells by membrane-anchored TGF-α in the absence of proteases. In the follicle this could provide a direct means of intracellular communication and consequently an additional mechanism for controlling follicle cell growth. Epidermal growth factor/TGF-α acts on target cells via interaction with specific 170-kDa transmembrane receptors which are tyrosine kinases. The phosphorylated C terminus is thought to unmask the catalytic domain of the receptor. A number of additional intracellular signaling pathways are activated within minutes of EGF binding to its receptor: activation of phospholipases, increase in intracellular calcium, activation of protein kinase C, and activation of serine/threonine kinases. Ultimately these individual changes result in altered cellular growth and differentiation (Mulheron and Schomberg, 1993).

Epidermal growth factor/TGF-α may be a primary mitogen of granulosa cells in most species (Lobb and Dorrington, 1992). Transforming growth factor-α/EGF has been also shown to stimulate maturation of granulosa cells (Tonetta and

DiZerega 1989) but inhibits follicular E_2 secretion in sheep (Webb et al 1994) Epidermal growth factor can inhibit production of both inhibin and progesterone by bovine granulosa cells but stimulates DNA and protein synthesis (Tonetta and DiZerega 1989) Epidermal growth factor binding is stimulated by FSH and EGF in turn enhances FSH binding (Urban and Veldhuis 1992) EGF inhibits FSH-induced aromatase and the formation of LH receptors in the rat and pig (Hsueh et al 1984) Prior exposure of granulosa cells to FSH has been reported to diminish the inhibitory effects of EGF on cell differentation (Paton and Collins 1992) In humans EGF and TGF-α are negative modulators of FSH-induced synthesis of E_2 and positive modulators of granulosa cell proliferation Their production by human follicles seems to decrease as follicular diameter enlarges (Gougeon 1986) In bovine granulosa cells in vitro EGF TGF-α and bFGF stimulate proliferation but inhibit steroidogenesis (FSH-induced E_2 secretion Rouillier et al 1997) and inhibin production (Skinner and Coffey 1988 Vernon and Spicer 1994) suggesting that they might act in vivo by enhancing growth and delaying terminal maturation of follicles (Monniaux et al 1997) Apoptotic cell death in granulosa cells of follicles selected for ovulation is prevented by the parecrine actions of EGF/TGF-α produced by thecal-interstitial cells or the autocrine and paracrine actions of bFGF synthesized by granulosa cells (Hsueh et al 1994)

Lobb and Domngton (1992) proposed a model for the role of transforming growth factors in ovarian physiology and follicular development In primordial follicles TGF-α of theca origin diffuses into the granulosa cell compartment and positively stimulates granulosa cell mitosis Granulosa cells in these follicles are not

fully differentiated and do not synthesize estrogens. While promoting granulosa cell growth, TGF-α may also maintain the granulosa cell in an undifferentiated state. At a latter stage of development (large antral follicles), granulosa cell mitosis declines and the cells begin to synthesize estrogens. TGF-β negatively affects granulosa cell growth while promoting gonadotrophin-induced steroidogenesis in these follicles. The interplay of the two growth factors leads at first to increase cell number and latter the augmentation of differentiate function. That culminates into a mature estrogen-active preovulatory follicle.

Insulin-like Growth Factors (IGFs) and IGF-binding Proteins (IGFBPs)

Insulin-like growth factors have direct effects on cultured ovarian cells. These effects include stimulation of granulosa cell mitogenesis, granulosa and luteal progesterone production, and thecal cell androgen production that appear similar among species (Spicer and Echternkamp, 1995). Administration of growth hormone stimulates growth of small antral follicles probably by an indirect mechanism involving IGF-I of endocrine origin. Terminal follicular growth is a strictly gonadotropin dependent process corresponding to initiation of follicular waves, selection of dominant follicles and terminal maturation of preovulatory follicles. The IGF family of growth factors may control the growth of small antral follicles in conjunction with FSH in the initiation of follicular waves. They are likely to be part of the players in the process of selection of the dominant follicle (Monniaux et al., 1997). The IGF system is composed of different elements: two ligands (IGF-I and IGF II), two receptors, and six or more IGF-binding proteins. IGFs function as

modulators of gonadotrophin action at the cellular level and stimulate granulosa and thecal cell proliferation and differentiation (Armstrong and Webb 1997). The type I receptor mediates most of the somatomedin-like actions of both IGF-I and -II. It is an $\alpha_2\beta_2$ tetramer structurally and functionally related to the insulin receptor. The affinity of this receptor for IGF-I is slightly higher than for IGF-II and much higher than for insulin. The type II receptor binds IGF-II and molecules that possess a mannose-6-phosphate residue such as lysosomal enzymes. The IGF-II receptor does not bind insulin and binds IGF-I with very low affinity (Monget and Monniaux, 1995).

The six IGFBPs bind IGF-I and II with high affinity. They are classified into two groups. IGFBP-1, -2, -4, -5 and -6 called 'small complex' which are present in serum and other fluids. Their molecular masses range between approximately 24 kDa and 35 kDa. Their concentrations in serum are either negatively (IGFBP-1 and -2) or not regulated by growth hormone. IGFBP-3 is the most predominant IGFBP in serum. It is present in a 150 kDa form or 'large complex'. The concentration of IGFBP-3 is positively regulated by growth hormone and IGF-I. IGFBPs increase the half life of IGFs and can both inhibit or potentiate IGF action at target cells. Their affinity for IGF-I and -II is of the same order of magnitude as the affinity of the type I receptor. Actions of IGF-I on follicular cells are mediated on granulosa and thecal cells by type I receptors (Giudice 1992). In cows, expression of the IGF-I receptor increases in small antral follicles (Wandji et al., 1992) whereas dominant follicles express abundant IGF-I and IGF-II mRNA (Yuan et al., 1998) but failed to express IGFBP-2 which is a binding protein associated with atresia (Monget and Monniaux,

1995). In sheep as in rats, pigs and humans, IGF-I stimulates both proliferation and differentiation of granulosa cells from follicles of 1-3 mm in diameter (Monget and Monniaux, 1995) and thecal cell steroidogenesis (Giudice, 1992; Paton and Collins, 1992). On follicles > 5 mm, IGF-I stimulates secretion of P_4 by granulosa cells (Monniaux and Pisselet, 1992). The mRNA encoding IGF-II has been detected in thecal tissue of bovine ovarian follicles (Armstrong and Webb, 1997) and localized in both granulosa and thecal cells of sheep (Leeuwenberg et al., 1995). The functional significance of these differences in the expression of mRNA encoding IGFs between species is unknown. It may reflect changes in the relative roles of IGF-I and -II that have evolved to fit the particular pattern of follicular development (Armstrong and Webb, 1997). As with the IGFs, the spatial expression of the binding proteins within ovarian follicles is species-specific. In cows (Armstrong et al., 1996; Yuan et al., 1995) and sheep (Besnard et al., 1996a), expression of mRNA encoding IGFBP-4 and -2 is restricted to theca and granulosa cells, respectively.

The release of IGFs from the IGFBPs is controlled by the action of specific IGFBP proteases. Specific IGFBP proteases have been detected in follicular fluid from ewes (Besnard et al., 1996b) and the amounts and activity of these enzymes change during folliculogenesis. Interaction between IGFs and other peptides (e.g. EGF, TGF-α) may be significant in regulating not only the proliferation but also the survival of granulosa cells (Paton and Collins, 1992). In cattle, doses of 100 ng/ml of IGF-I either had no effect or inhibited basal and FSH-induced E_2 production by granulosa cells collected from small (< 5 mm) follicles, and was a weak stimulator

of FSH-induced E_2 production by granulosa cells from large follicles (< 8 mm). Insulin, IGF-I and IGF-II stimulate progesterone production by bovine granulosa cells and concomitant with FSH treatment, enhances the stimulatory effect of IGF-I. Insulin and IGF-I synergize with LH to promote thecal cell androgen production, and bovine thecal cells appear much more sensitive to insulin (Spicer et al., 1993).

No changes in follicular fluid concentrations of IGF-I were detected in first wave dominant (Badinga et al., 1992; de la Sota et al., 1996) and subordinate (de la Sota et al., 1996) follicles collected on days 5, 9 and 12 of the estrous cycle. In contrast, Echternkamp et al. (1994) reported higher concentrations of IGF-I in follicular fluid of large E_2 active follicles compared to E_2 inactive and small follicles. De la Sota et al. (1996) and Echternkamp et al. (1994) reported changes in the absolute amounts and proportions of IGFBP-2, -4 and -5. They were at low levels in E_2 active dominant follicles but are at high levels in atretic dominant follicles which may reduce the concentrations of free IGF-I and -II available in follicular fluid to exert their trophic activity on granulosa cells.

Manipulation of The Follicular Dynamics in Cattle

The failure of hormonal treatments to control follicular development and superovulation in women and domestic animals in a very precise manner gives evidence of significant gaps in the understanding of the mechanisms that regulate development and function of ovulatory follicles (Fortune et al., 1988). Furthermore, the mechanisms that control the ovulatory quota in different domestic animals are not completely understood. It is clear that the development of medium- sized and

large follicles in cattle occurs in waves. The regularity of the patterns allows experimental manipulations designed to determine how these patterns are controlled (Fortune et al., 1991).

Control of follicular development in cattle involves the removal of the suppressive effect of the dominant follicle to allow emergence of a new follicular wave at a specific time after treatment. Different approaches have been followed to control follicular dynamics in cattle. Hormonal approaches have been directed to cause luteinization or atresia of the follicles present at the time of treatment (Bo et al., 1994). This has been accomplished by using hCG (Rajamahendran and Sianangama, 1992; Diaz et al., 1993) or GnRH (Macmillan and Thatcher, 1991; Schmitt et al., 1996b) to induce follicle luteinization or ovulation, or by progestogens and E_2 to cause atresia of the dominant follicle (Bo et al., 1994).

A multitude of experimental approaches have been used to control follicle dynamics in cattle, including treatments with hCG (Diaz et al., 1993; Schmitt et al., 1996b; Sianangama and Rajamahendran, 1996), GnRH analogs (Macmillan and Thatcher, 1991; Rusbridge et al., 1992; Schmitt et al., 1996a), electrocautery of the dominant follicle during its growing phase (Ko et al., 1991; Adams et al., 1993b), ultrasound-guided follicle aspiration (Bergfelt et al., 1994), pretreatment with FSH given early in the estrous cycle to increase the number of embryos recovered after a superovulatory treatment (Goulding et al., 1991; Gray et al., 1992), and treatment with high doses of P_4 during the growing phase of the dominant follicle to suppress the dominant follicle and hasten the emergence of the next wave (Adams et al., 1992a; Burke et al., 1994).

Hormonal Manipulation Human Chorionic Gonadotropin and Gonadotropin Releasing Hormone

It has been demonstrated that the first wave dominant follicle can be ovulated after administration of Gonadotropin Releasing Hormone (GnRH Rusbridge et al 1992 Schmitt et al 1996a) or human Chorionic Gonadotropin (hCG) Human Chorionic Gonadotrop-n is a glycoprotein with LH-like activity that promotes ovulation of the first wave dominant follicle and formation of an accessory corpus luteum (CL) when administered on d 4 (Breuel et al 1989 Price and Webb 1989) day 5 (Walton et al 1990 Schmitt et al 1996a 1996b) day 6 (Fricke et al 1993) day 7 (Breuel et al 1989 Rajamahendran and Sianangama 1992 Sianangama and Rajamahendran 1992 1996) day 10 (Breuel et al 1989) or day 14 to 18 (Price and Webb 1989) of the estrous cycle

Both GnRH and hCG have the ability to induce an accessory CL during the early luteal phase of the estrous cycle in cattle which could have some practical applications Establishment and maintenance of pregnancy as well as embryo survival in cattle are related to the ability of the CL to secrete P_4 (Thatcher et al 1994b) Subluteal levels of P_4 could be a factor that contribute to embryo losses (Thatcher et al 1994b) Also the rate of increase in plasma P_4 during the early luteal phase could play a role in establishment of pregnancy (Shelton et al 1990) Progesterone supplementation after insemination in heat stressed cows may correct deleterious effects of impaired luteal function on pregnancy rates Inducing formation of an additional CL during the luteal phase of the estrous cycle with an

injection of either GnRH or hCG could be a strategy to increase concentrations of P_4 in plasma during the period when maternal recognition of pregnancy occurs (Schmitt 1995)

Ablation of the Dominant Follicle

There is a potential for artificial control of follicular dynamics using ablation procedures Two types of ablation procedures have been tried electrocauterization (Ko et al 1991 Adams et al 1993a) and ultrasound-guided transvaginal follicle aspiration (Bergfelt et al 1994 Stubbings and Walton 1995) The first one is an invasive method and not repeatable on a within cow basis whereas follicle aspiration is a noninvasive and repeatable procedure that can be used for the retrieval of oocytes for in vitro fertilization (Pieterse et al 1988 1991a 1991b)

It has been shown that electrocauterization (Ko et al 1991 Adams et al 1993a) of the dominant follicle during its growing phase hastened emergence of the next follicular wave Ko et al (1991) showed that cauterization of first wave dominant follicle on days caused an early emergence of wave 2 and increased the incidence of 3-wave interovulatory intervals

Follicle ablation via ultrasound-guided follicle aspiration at random (all follicles ≤ 5 mm) stages of the estrous cycle synchronized subsequent follicular wave emergence and resulted in a high degree of synchronized ovulations in heifers following $PGF_{2\alpha}$ induced luteolysis (Bergfelt et al 1994)

Superstimulation

The objective of ovarian superstimulatory treatment in cattle is to obtain the maximum number of viable embryos by stimulating growth and subsequent ovulation of competent antral follicles with exogenous gonadotropins (Adams 1994). However, the variable and unpredictable superovulatory response of the donor animal has remained one of the most limiting factors to successful embryo transfer (Armstrong, 1993). To date all methods of inducing superovulation in cows have involved administration of exogenous gonadotropic hormones, crude or purified hypophyseal extracts, such as porcine follicle-stimulating hormone and luteinizing hormone, equine chorionic gonadotropin and human menopausal gonadotropin (Mapletoft et al., 1994).

Many reports have been published on dosage regimes and types of gonadotropin preparations for ovarian superstimulation (Elsden et al., 1978; Murphy et al., 1984; McGowan et al., 1995; Lerner et al., 1986; Pawlyshyn et al., 1986; Armstrong, 1993) but timing of treatment with respect to monitored follicular wave development has received little attention (Adams et al., 1993b). Animal variability is important as a determinant of superovulatory response. The ovarian status of the donor at the time of hormone treatment appears to be a major determinant of the superovulatory response (Monniaux et al., 1983). In recent years, research has been focused on making responses more predictable by administering superstimulation treatments with specific regard to the status of follicular development at initiation of treatment (reviewed by Armstrong, 1993). Results from

different studies (Pierson and Ginther, 1988; Grasso et al., 1989; Guilbault et al., 1991) suggested that the superovulatory response is affected by the presence of a large dominant follicle (Pierson and Ginther, 1988; Guilbault et al., 1991; Adams et al., 1993b; Wolfsdorf et al., 1997). Superovulation may be induced with equal efficacy when treatment is initiated during the time of the first or second follicular waves when a dominant follicle is present in the ovaries, and the superovulatory response is enhanced if treatment is initiated at the time of wave emergence, before the time of follicle selection (Adams, 1994). It has been shown (Wehrman et al., 1996) that FSH treatment in presence of a persistent dominant follicle resulted in follicles that were unable to respond to the preovulatory surge of LH.

Priming doses of FSH given at the beginning of the cycle have been shown to increase the superovulatory response to FSH treatments (Rajamahendran et al., 1987; Ware et al., 1987; Touati et al., 1991). Others have failed to show the beneficial affect of early priming (Grasso et al., 1989; Gray et al., 1992). The idea of the priming is to increase the number of follicles in the FSH-responsive pool. It is likely that priming with other hormones, such as PMSG, which possesses both FSH and LH activity, could increase the number of FSH-responsive follicles (Monniaux et al., 1984).

Passive immunization against inhibitors of gonadotropin secretion may also be a means of increasing ovulatory rates by short-term elevation of levels of endogenous follicle-stimulating hormone (Mapletoft et al., 1994). It has been shown that passive immunization of ewes against steroid free-follicular fluid or against synthetic inhibin peptides increased ovulation rates two- to four-fold (O'Shea et al.,

1991 Schanbacher et al 1991) Ovulation rate and prolificacy in the cow are more difficult to manipulate by inhibin vaccination (O'Shea et al 1994) However there are reports that immunization of heifers or cows against steroid free follicular fluid increases ovulation rate (Price et al 1987 Glencross et al 1992 Morris et al 1993 Scanlon et al 1993) and number of transferable embryos (Alvarez et al 1997)

In summary there are several factors involved in the control of ovarian follicular dynamics in cattle and some of the mechanisms that control ovarian follicular growth have been elucidated but still there are more that are not completely understood as an example factor(s) and mechanisms that control dominance during a follicular wave A set of experiments was designed and performed with the aim of this study to identify the factor (s) and mechanisms that control follicular dominance in cattle Understanding these mechanisms will be important for the design of programs for synchronization of the estrous cycle and ovulation and for superovulation and embryo transfer

CHAPTER 3
HUMAN CHORIONIC GONADOTROPIN INDUCED ALTERATIONS IN OVARIAN FOLLICULAR DYNAMICS DURING THE ESTROUS CYCLE OF HEIFERS

Introduction

The understanding of factors controlling ovarian folliculogenesis should lead to development of new and more precise methods of controlling reproductive cycles in livestock. One aspect of follicular dynamics is the process of continual growth and regression of antral follicles that leads to development of the preovulatory follicle (Lucy et al. 1992). One to four waves of follicular growth and development occur during a single estrous cycle in heifers and cows and the preovulatory follicle is derived from the last wave. During each wave, development of antral follicles ≥ 2 mm proceeds through stages of follicular recruitment, selection and dominance (Savio et al., 1988, 1990; Lucy et al., 1992; Fortune, 1994; Ginther et al. 1996). Development of the first dominant follicle (DF) is the most consistent event related to follicular dynamics during the estrous cycle in heifers and cows. The first dominant follicle can be identified retrospectively by ultrasonography between day 2 and 4 of the estrous cycle (Savio et al., 1988, 1990; Sirois and Fortune, 1988; Driancourt et al., 1991; Ginther et al., 1996).

Human chorionic gonadotropin (hCG) is a glycoprotein with LH-like activity

that promotes ovulation of the first wave DF (FWDF) and formation of an accessory corpus luteum (CL) when administered on day 4 (Breuel et al 1989 Price and Webb 1989), day 5 (Walton et al 1990 Schmitt et al , 1996a 1996b) day 6 (Fricke et al 1993) day 7 (Breuel et al 1989 Rajamahendran and Sianangama 1992 Sianangama and Rajamahendran 1992 1996) day 10 (Breuel et al 1989) or day 14 to 16 (Price and Webb 1989) of the estrous cycle With formation of an accessory CL, there is a subsequent increase in concentrations of plasma progesterone (P_4; Breuel et al , 1989 Fricke et al 1993 Schmitt et al 1996a) Only one study has characterized ovarian follicular dynamics during this period (Sianangama and Rajamahendran 1996) The objective of the present experiment was to characterize hCG induced alterations in ovarian follicular and CL dynamics in heifers when hCG was injected on day 5 of the estrous cycle

Materials and Methods

Seventeen sexually mature Holstein heifers were selected from the dairy herd at the University of Florida Heifers were kept in a lot with minimal pasture and fed peanut hay and corn silage Estrous cycles were synchronized following removal of a norgestomet ear implant (6 mg norgestomet implant without injection of the norgestomet/estradiol valerate solution Synchromate-B Sanofi Animal Health Inc., Overland Park KS) that had been in place for 7 days and injection of prostaglandin (PG) $F_{2\alpha}$ (25 mg i.m. Lutalyse Pharmacia & Upjohn Co , Kalamazoo MI) on day 6 or 1 day before withdrawal of the implant Brightly colored enamel-based paint (Impervo Benjamin Moore Montvale NJ) was applied to the tailhead

of each heifer at the time of $PGF_{2\alpha}$ administration. When the ear implant was removed, the paint strip was covered with a contrasting color of chalk (All-Weather® Painstick® Lake Chem Co., Chicago, IL; Van Cleeff et al., 1996). The extent in cover of tail paint and chalk (TPC) was scored based on a modification of the method of Macmillan et al. (1988) using a scale of 5 (no signs of estrus and full presence of paint and chalk) to 0 (standing estrus and absence of paint and chalk; Van Cleeff et al., 1996). Scoring of the TPC and visual detection of estrus were performed twice daily (0600 to 0700 and 1800 to 1900) for 3 days after removal of the implant. On day 5 of the estrous cycle (day 0= day of estrus), heifers (n=6) were treated with hCG (1 000 IU given i.v.; 2 000 IU given i.m., Stans Laboretones, Phoenix, AZ, n=6; Schmitt et al., 1996a) or i.m. injection of saline (control group, n=7). A real time ultrasound scanner equipped with a linear array 7.5 MHz transrectal transducer (Equisonic LS 1000, Tokyo Keiki Co., LTD, Tokyo, Japan) was used to monitor follicular development daily from day 3 of the estrous cycle until the next ovulation (first cycle). During the following estrous cycle (second cycle), follicular development and CL growth were monitored every other day until estrus. Ovarian structures (follicles $\geq$ 2 mm and CL) were measured, and their relative positions recorded on ovarian maps drawn during the examination. Blood samples were taken daily during the first and second estrous cycles. The dominant follicle for each follicular wave was defined as the largest newly emerged ovarian follicle that was 2 mm greater than the second largest follicle and grew linearly (Sirois and Fortune, 1990).

Blood samples (10 ml) were collected daily via venipuncture from the jugular

vein into heparinized tubes Blood samples were stored in an ice bath plasma separated by centrifugation (1 800 x g for 15 minutes) and stored at -20 °C until assayed for P_4 and estradiol (E_2) Concentrations of P_4 in plasma were measured by radioimmunoassay (Knickerbocker et al 1986) Sensitivity of the assay was 0 15 ng/ml Intra- and inter assay coefficients of variation were 4 6 and 13.2% respectively Concentrations of E_2 from day 8 before ovulation until day 7 of the second cycle were measured by a radioimmunoassay described by Tortonese et al (1990) and modified by Bedinga et al (1992) Sensitivity of the assay was 1.0 pg/ml and the intra- and inter-assay coefficients of variation were 6.9 and 19 9% respectively

Statistical Analyses

Data were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS 1988) The following definitions were used to characterize each follicular wave a) day of emergence of DF was the day when the DF first appeared in the ovary and was at least 5 mm in size b) day of selection of DF was the day of the cycle when the difference between DF and second largest follicle (subordinate) was $\geq$ 2 mm, c) day of maximum recruitment was the day when maximum number of class 2 follicles (6-9 mm) was present in the ovary and d) duration of a follicular wave was the difference between day of emergence of the DF of two consecutive waves

The mathematical models used to analyze size of dominant and subordinate follicles and number of follicles within discrete size classes (Class 1 2 to 5 mm

Class 2 6-9 mm Class 3 ≥ 10 mm) included effects of treatment, cow(treatment), day of the wave (wave II days 1 to 18 [days relative to day of emergence of second wave DF] and wave III days 1 to 13 [days relative to day of emergence of third wave DF]), and the interaction of day of the wave by treatment. The main effect of treatment was tested using the mean square of cow (treatment) as the error term. Tests for homogeneity of regression equations were performed on equations for follicular diameter versus day of wave for dominant and subordinate follicles of the second and third follicular waves. These analyses were performed as described by Wilcox et al. (1990).

The same analytical approach was used to analyze plasma concentrations of P_4 during the first and second estrous cycles for hCG and control heifers; plasma concentrations of E_2 during the peri-ovulatory period; sizes of the original and induced CL, and patterns of growth of original CL in hCG-treated and control heifers. However, day of cycle (P_4), days from ovulation (E_2), and days after ovulation (original vs. induced CL and comparison of original CL growth for both groups) were used for these analyzes. The mathematical model used to analyze the follicular dynamics and plasma concentrations of P_4 between heifers with two and three follicular waves during the first and second estrous cycles did not include hCG-treated heifers during the first estrous cycle. The model included effects of wave, cycle, cow(wave-cycle), day, and interactions wave by day, cycle by day, and wave by cycle by day. The main effects of wave and cycle were tested using the mean square of cow(wave-cycle) as the error term.

Duration of the second and third follicular waves in all heifers with three-wave

cycles were analyzed using a mathematical modal that included the effects of treatment, wave(second or third wave) and the interaction of treatment by wave. The frequencies of accessory CL and estrous cycles of two versus three follicular waves between treatments were compared using Chi-square analyses

Results

Corpora lutea and plasma progesterone

Treatment with hCG at day 5 induced ovulation and formation of an accessory CL in all six heifers of the hCG group while none of the control heifers ovulated the first wave dominant follicle (χ^2= 13, P < 0.01). Progesterone concentrations increased from 0.9 ng/ml on day 1 to 6.4 ng/ml on day 6 and did not differ (P > 0.10) between heifers in both treatments. However, by day 9, P_4 concentrations were greater (P < 0.01, Figure 3-1A) for heifers of the hCG group (15.3 ng/ml) than for control heifers (11.1 ng/ml) and remained elevated until day 17. Maximal concentrations of P_4 were observed on day 15 (hCG group, 20.4 vs control group, 12.9 ng/ml). On day 19.5 and 20.3 of the estrous cycle, there was a 50% decrease in P_4 concentrations for hCG and control groups, respectively, indicating that luteolysis had been initiated. P_4 concentrations were < 1 ng/ml on day 21 for both groups. Concentrations of P_4 during the following estrous cycle did not differ between groups (P > 0.10). The profile was the same in both groups, reaching maximal concentrations on day 13 (11.3 vs. 10.6 ng/ml, for hCG and control groups, respectivaly, Figure 3-1B).

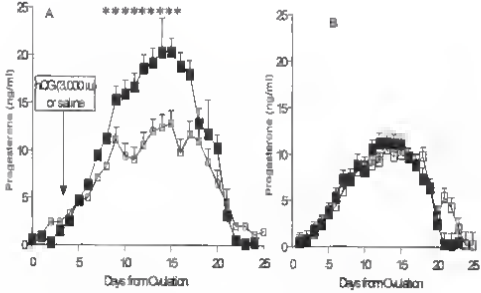

Figure 3-1 Plasma concentrations of progesterone (LSM ± SEM) during the first (A) and second (B) estrous cycle in control (□) and hCG-treated (■) heifers. Asterisk (*) indicated significant differences (P < .01) between mean concentrations within day

There was a difference (P < 0.01; Figure 3-2) in concentrations of P_4 between heifers with two (13 heifer-cycle cells) and three follicular waves (8 heifer-cycle cells). This comparison was made on all heifers except hCG-treated heifers of the first experimental cycle. Concentrations of P_4 from day 13 to 20 were lower in heifers having two-wave cycles compared with three-wave cycles. There was a 50% decrease in P_4 concentrations by day 17.7 in heifers with two waves and day 21.3 in heifers with three follicular waves, which is indicative that the luteolytic mechanism from the uterus was active earlier in heifers with two follicular waves (Figure 3-2).

Dynamics of CL development differed (P < 0.001) between groups due to

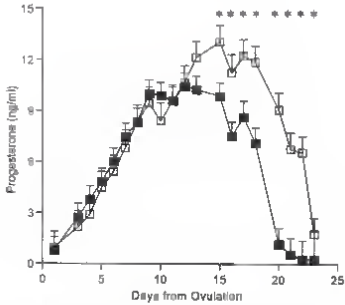

Figure 3-2 Plasma concentrations of progesterone (LSM ± SEM) for heifers with two (■) and three (□) follicular waves during the first and second estrous cycles Asterisk (*) indicates differences ($P < .01$) between mean concentrations within day

Induction of accessory CL In the hCG-treated heifers (Figure 3-3) In hCG-treated heifers growth of the original and induced CL did not differ ($P > 0.10$) from day 3 to day 8 after ovulation The accessory CL was smaller ($P < 0.01$) than the original CL and regressed concurrently with the original CL in hCG-treated heifers There was no difference ($P > 0.10$) in average size between original CL of hCG-treated and control heifers (Figure 3-3A and 3-3B) but there was a treatment by day interaction ($P < 0.001$) for the pattern of growth of the original CL in hCG-treated and control heifers Between day 3 and 11 of the estrous cycle original CL of hCG-treated heifers grew to a larger size following injection of hCG

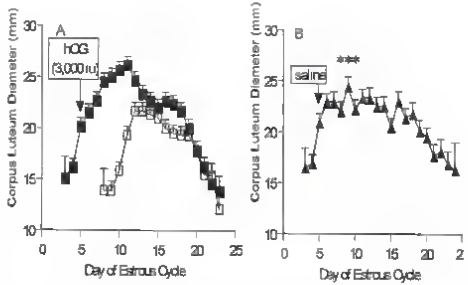

Figure 3-3 Growth of the original (■) and induced (□) corpora lutea in hCG-treated heifers (A) and original (▲) corpus luteum in control (B) heifers during the first estrous cycle. Asterisk (*) indicates differences ($P < 0.01$) between least squares means within day.

Follicular Dynamics

All hCG-treated heifers had three follicular waves during the first experimental estrous cycle (Figure 3-4). In the control group, in contrast, three heifers had three follicular waves (Figure 3-5A) and four had two follicular waves (Figure 3-5B). The frequency of three follicular waves was greater ($\chi^2 = 4.95$, $P < 0.05$) in the hCG group.

The second wave dominant follicle emerged earlier in hCG-treated heifers (day 7.3; Figure 3-4) compared with the control group (day 10.4; $P < 0.01$; Figure 3-5A and 3-5B) and reached 10 mm in diameter on day 10 of the estrous cycle for hCG-treated heifers compared with 13.6 day for heifers in control group with either

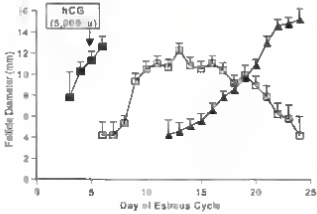

Figure 3-4 Growth of the first (■) second (□) and third (▲) dominant follicles in hCG-treated heifers (n=6) during the first estrous cycle

two or three follicular waves In both groups the second wave dominant follicle was selected on day 3 4 of the wave and day of maximum recruitment was day 3 7 A treatment by wave interaction was detected ($P < 0.01$) for duration of waves for heifers with three follicular waves during the first cycle (Figure 3-4 and 3-5A) In the control group the second and third follicular waves lasted 8 3 ± 0 8 days and 5 3 ± 0 9 days respectively For the hCG group duration of the second follicular wave was shorter (6 3 ± 0 7 days) possibly due to greater P_4 concentrations whereas duration of the third wave was longer (9 2 ± 0 7 days) than in the control group Maximum diameter of the second wave dominant follicle was smaller ($P < 0.01$) in hCG treated heifers (12.5 ± 0 8 mm Figure 3-4) than in control heifers (15 6 ± 0 8 mm Figure 3-5A) with three-wave cycles. Associated with the differences in duration of third wave follicles (hCG 9 2 days > control 5 3 days $P < 0.01$) were differences in follicular dominance as measured by growth patterns of the largest subordinate follicle The subordinate follicle of the hCG group decreased in size

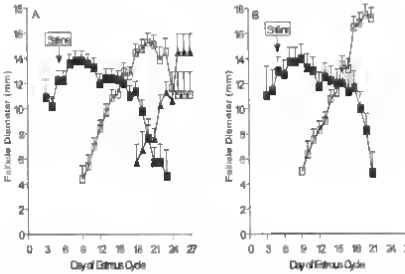

Figure 3 5 Growth of the first (■) second (□) and third (▲) dominant follicles in control heifers with three (n= 4 A) and two (n= 3 B) follicular waves during the first estrous cycle

during the 6 days of the preovulatory period (b= -0 007 mm/day) In contrast, size of the subordinate follicle of the control group increased during this period (b= 0 47 mm/day P < 0 01) This may be a reflection of a more developed and active preovulatory dominant follicle in hCG-treated heifers that suppressed growth of the subordinate follicle The third dominant follicle emerged on day 14 7 and 18 of the estrous cycle for heifers of the hCG and control groups respectively selection occurred on day 3 7 of the third wave for both groups

During the second estrous cycle one hCG-treated heifer had three follicular waves and five had two follicular waves while three saline-treated heifers had three follicular waves and four heifers had two follicular waves A total comparison of two- and three-wave cycles excluding first cycles for hCG-treated heifers was made

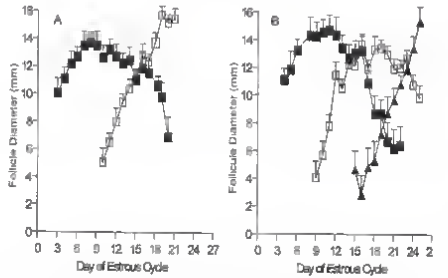

Figure 3-6. Growth of the first (■), second (□), and third (▲) dominant follicles in heifers with two (n=13, A) and three (n= 8, B) follicular waves during the first and second estrous cycle. Heifers treated with hCG during first estrous cycle are not included

utilizing first and second estrous cycles. The second wave dominant follicle in two-wave cycle heifers had a longer life span (11 days) than the second dominant follicle of three-wave cycle heifers (6 days; Figure 3-6A and 3-6B). Similarly, there was a significant wave by day interaction ($P < 0.01$) for size of the ovulatory follicle in heifers with two and three follicular waves. The ovulatory follicle of heifers with two-wave cycles entered a plateau phase for 3 days before ovulation, whereas growth of the ovulatory follicle of heifers with three-wave cycles was linear until ovulation.

Estradiol-17β Concentrations

Plasma concentrations of E_2 from day 6 before ovulation until 7 days post ovulation did not differ ($P > 0.10$; Figure 3-7A) between treatments. Heifers treated

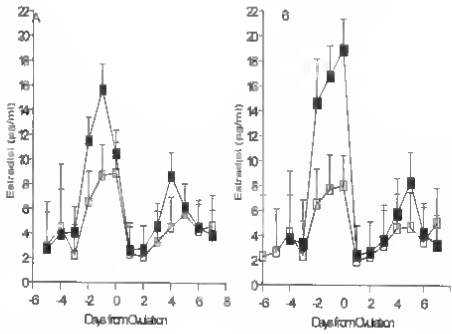

Figure 3-7 Plasma concentrations of estradiol 17ß (LSM ± SEM) in hCG-treated (■) and control (□) heifers (A) and control heifers with two follicular waves (■; n= 4; B) and three follicular waves (□ n=3 B) during the peri-ovulatory period

with hCG tended to have greater E_2 concentrations (15 6 pg/ml) than heifers of the control group (8 2 pg/ml) on the day before ovulation This trend was due to one heifer that had very high concentrations of E_2 Following ovulation plasma concentrations of E_2 increased to 8 7 pg/ml on day 4 for hCG treated heifers and to 5 6 pg/ml on day 5 for the control group This increase in E_2 did not differ between treatments and coincided with growth of the first wave dominant follicle before the luteal phase increase in concentrations of P_4 Plasma concentrations of E_2 tended ($P < 0.10$) to be greater from day -3 until day of ovulation (day 0 Figure 3-7B) in control heifers with two follicular waves (n=2) than in control heifers with three follicular waves (n=3) This coincides with the fact that the second dominant

follicle was present longer on the ovary. The ovulatory follicle in control heifers with two follicular waves reached a size ≥ 10 mm on day 13.5 of the estrous cycle versus day 22.3 for control heifers with three follicular waves (Figure 3-5A and 3-5B).

Duration of Estrous Cycles

Duration of the first estrous cycle for hCG group and control group was 22.8 ± 0.9 and 22.1 ± 0.8 days (P > 0.10) and 21.8 ± 1.0 and 22.7 ± 1.0 days (P > 0.10) for the second estrous cycle. Analysis of the first and second experimental cycles, excluding the first estrous cycle of hCG-treated heifers, indicated that estrous cycles with two waves of follicular development were shorter (20.5 ± 0.5 days, P > 0.01) than those of three-wave estrous cycles (24.6 ± 0.7 days).

Discussion

Cows and heifers exhibit two or three successive waves of follicular development during the estrous cycle (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a, 1989b). In this study, the FWDF present at day 5 ovulated in response to hCG and formed a CL which is in agreement with others (Breuel et al., 1989; Price and Webb, 1989; Walton et al., 1990; Rajamahendran and Sianangama, 1992; Sianangama and Rajamahendran, 1995; Schmitt et al., 1996a, 1996b). Previous work indicated that the response was affected by stage of the estrous cycle when hCG was given. Induction of an accessory CL was greater on days 4 to 7 and days 14 to 16 than between days 8 to 13 of the estrous cycle (Price and Webb, 1989). This is due likely to transitional stages in follicular status within

the first (Badinga et. 1992 Thatcher et al 1996) and second follicular waves that are either responsive or nonresponsive to hCG Schmitt et al (1996a) reported that the greater growth of original CL in hCG-treated compared to control heifers could be due to a luteotropic effect of hCG on development of the original CL after day 5

In the present study hCG acutely altered follicular dynamics by ovulating the first wave dominant follicle on day 5 All hCG-treated heifers had three follicular waves during the experimental estrous cycle Elimination of the first wave dominant follicle via hCG induction of ovulation leads to an earlier recruitment and emergence of the second-wave DF Badinga et al (1992) reported that removing the ovary bearing the DF increased FSH leading to follicular recruitment..thus removal of the FWDF by hCG-induced ovulation may have had the same effect in this study Ko et al (1991) reported that destruction of the FWDF later during the wave is followed by an earlier recruitment of the next wave A comparable effect appears to occur with elimination of the DF via induction of ovulation with hCG

Although an earlier emergence of the second wave follicle occurred in hCG-treated heifers maximum size of the second wave follicle was reduced and had a shorter lifespan This was attributed to the greater P_4 concentrations associated with induction of an accessory CL in this group Indeed greater concentrations of P_4 reduce growth of the DF and induce follicle turnover (Savio et. 1993 Burke et al. 1994 Thatcher et al 1994, Kinder et al , 1996) due to reduction in LH secretion (Kinder et al 1996) Functional duration of the third wave follicle was increased because of a shorter life span of the second-wave follicle in hCG-treated heifers, Indeed..e greater decrease in size of the subordinate follicle during the preovulatory

period for the third wave dominant follicle of the hCG group was detected compared to that of the preovulatory subordinate follicle of three-wave cycles in the control group. Regardless of day of emergence or duration of a wave, dominant follicles were selected between day 3 and 4 of the respective wave. Ginther et al. (1996) reported that between day 0 and 4 of a wave, the dominant and largest subordinate follicles diverge gradually in diameter.

Administration of hCG during the luteal phase of the estrous cycle increased P_4 concentrations in blood and in some cases extended duration of the estrous cycle (Seguin et al. 1977, Breuel et al. 1989, Walton et al. 1990, Rajamahendran and Sianangama 1992, Fricke et al. 1993, Schmitt et al. 1996a). This effect appears to be somewhat dependent on presence of a functional CL because hCG administered on day 1 (early luteal phase) or day 17 (late luteal phase) of the estrous cycle had no effect on serum P_4 concentrations (Seguin et al. 1977, Breuel et al. 1989). In this experiment, administration of hCG on day 5 increased plasma P_4 concentrations from day 9 to 17. The increase in P_4 concentrations was due most likely to additional P_4 secretion by accessory CL (Schmitt et al. 1996b) at this stage of the estrous cycle as opposed to a stimulation of the original CL (Fricke et al., 1993). Based upon diameter of the induced CL and the temporal increase in plasma P_4 concentrations, induced CL appear to be functional but smaller than the original CL. Sianangama and Rajamahendran (1996) demonstrated that the CL induced by hCG given on day 7 of the bovine estrous cycle is functional but appears to be smaller and secretes less P_4 than the spontaneous CL of similar age.

Because concentrations of plasma P_4 during the second cycle were similar

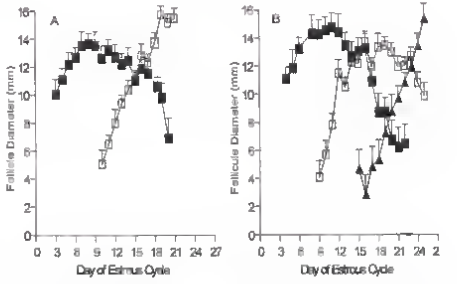

Figure 3-6 Growth of the first (■) second (□) and third (▲) dominant follicles in heifers with two (n=13 A) and three (n=8 B) follicular waves during the first and second estrous cycle. Heifers treated with hCG during first estrous cycle are not included

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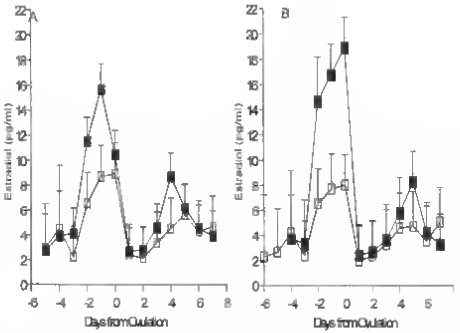

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Because concentrations of plasma P_4 during the second cycle were similar

between groups luteotrophic effects and alterations in dynamics of follicular growth observed in the first cycle after hCG administration failed to elicit any carry-over effects that influenced CL development and function of the second cycle. Similarly Breuel et al. (1989) did not observe any effect of hCG on CL formed during subsequent cycles.

Greater concentrations of plasma P_4 in heifers with three spontaneous follicular waves during the cycle could contribute to a greater turnover of the second wave dominant follicle as a consequence of decreased LH pulsatility (Kinder et al. 1996). Estrous cycles were significantly longer for three-wave cycles and growth characteristics of the ovulatory follicle were different between three-wave versus two-wave follicle cycles. The duration of the luteal phase appears to determine at least in part the number of follicular waves during an estrous cycle (Fortune 1994). Estrous cycles with three follicular waves have slightly but significantly longer luteal phases than cycles with two waves (Ginther et al. 1989b; Fortune 1993). We choose to interpret that two-wave follicular cycles induce an earlier CL regression compared to three-wave cycles. Contributing to this is presence of an active estrogenic follicle when CL regression is initiated. This could be determined partially by high plasma P_4 concentration leading to a turnover of the second wave follicle versus persistence of a potentially estrogenic second wave follicle in luteal phase with lower progesterone concentrations.

Third wave ovulatory follicles underwent a linear growth until ovulation versus a distinct plateau phase for the second wave ovulatory follicle. Whether fertility differs to insemination following ovulation of two versus three wave ovulatory

follicles needs to be examined Any differences in development of the third wave dominant follicle between hCG and control heifers were not reflected in differences of plasma E_2 Howard et al (1990) reported that hCG (10 000 IU, i m) administered on day 10 caused a transient increase in E_2 with normal concentrations observed during the subsequent pre-estrual/estrual period The tendency of higher concentrations of E_2 in control heifers with two waves versus three waves could reflect the presence of a more persistent estrogenic dominant follicle that is less fertile than a less estrogenic third wave dominant ovulatory follicle

An additional factor that may regulate fertility is the occurrence of two- versus three-wave cycles after insemination Heifers with two-wave cycles produced a potentially estrogenic preovulatory follicle (≥ 10 mm) as early as day 13 5 versus day 17 7 for three-wave cycles Since the signal mechanism induced by the conceptus to extend the CL lifespan occurs between 15 to 17 days it would appear that cows with three-waves cycles would be less likely to potentially antagonize the antiluteolytic mechanism Injection of estradiol stimulates uterus secretion of $PGF_{2\alpha}$ (Knickerbocker et al 1982) and cautenzation of follicles extends CL lifespan (Fogwell et al 1985) Thus heifers with three-wave cycles may conceivably have a higher rate of embryo survival Reproductive management systems to optimize ovarian follicular development in either the preovulatory period or following insemination to improve conception rate and embryo survival warrant further investigation

Implications

Injection of hCG (3000 IU) on day 5 of the estrous cycle ovulated the FWDF and resulted in estrous cycles with three follicular waves. Concentrations of P_4 during the luteal phase were elevated substantially and recruitment of the second wave follicle occurred earlier but had a shorter duration of dominance. The hCG-induced alterations in follicle development (induction of three-wave cycles versus two-wave cycles) when given on day 5 of the estrous cycle may be a means to optimize conception rates when given in the cycle before insemination or to increase embryo survival when given on day 5 after insemination. The latter response may also be associated with increased P_4 secretion that favors enhanced embryo survival.

The alteration in follicular dynamics induced by ovulation of the first wave dominant follicle with an hCG injection on day 5 suggests that there is a factor (s) in the DF that plays a role in dominance. Additional research is needed to elucidate the effect of the presence or absence of the first wave DF on the response to a superovulatory treatment.

CHAPTER 4
EFFECTS OF FSH P ON FOLLICULAR DYNAMICS AND OVARIAN RESPONSE TO A SUPEROVULATORY TREATMENT FOLLOWING ASPIRATION OF A FIRST WAVE PERSISTENT DOMINANT FOLLICLE

Introduction

Endocrinological and ultrasonographical investigations have demonstrated that growth of the dominant follicle (DF) during the bovine estrous cycle occurs primarily as two or three waves. A DF is defined as a large ovarian follicle that is recruited and selected during a follicular wave, has an initial linear growth rate, and is at least 2 mm greater than the largest subordinate follicle (Sirois and Fortune, 1990). During each wave of follicular growth, the DF develops and suppresses growth of subordinate follicles and inhibits recruitment of a new follicular wave (Ireland and Roche, 1987; Sirois and Fortune, 1988; Savio et al., 1988, 1990; Lucy et al., 1992).

One goal of superovulation is to suppress atresia in a greater number of follicles, thereby allowing them to attain preovulatory status (Mapletoft et al., 1994). There is a high degree of unpredictability in superovulatory responses that decreases the efficiency and profitability of embryo transfer programs. Monniaux et al. (1983) reported that ovarian status at the time of the superovulatory regime can account for a large portion of the variability in superovulatory response. For

example recent reports suggest that the presence or absence of a DF can affect superovulatory responses in cattle. It has been shown that superovulatory responses are reduced in animals treated with FSH in the presence of a DF compared with animals without a DF at the onset of the superovulatory treatment (Grasso et al. 1989; Guilbault et al., 1991; Huhtinen et al., 1992; Bungartz and Niemann, 1994; Wolfsdorf et al., 1997).

The ovarian response in animals with a DF at the time of superovulation induction can be improved by removing the DF by transvaginal aspiration (Armstrong, 1993). This procedure also is used to collect immature bovine oocytes repeatedly from the same donor (Pieterse et al., 1991). Immediately after elimination of the DF there is a rise in FSH concentrations that induces recruitment of a new follicular wave (Adams et al., 1992b; Bedinga et al., 1992). However, it is possible that presence of a DF may alter ovarian responsiveness to exogenous gonadotropins. Follicles smaller than 8 mm in diameter are recruited during gonadotropin treatment (Monniaux et al., 1983; Grasso et al., 1989), and presence of a DF may inhibit the recruitment process (Wolfsdorf et al., 1997).

It has been shown (Adams et al., 1992a; Savio et al., 1993a, 1993b; Stock and Fortune, 1993; Burke et al., 1994) that progesterone (P_4) plays an important role in growth and turnover of the DF. A negative feedback of P_4 on LH pulse frequency is a mechanism by which P_4 effects follicular growth and the demise of the first wave DF (Abeyawardene and Pope, 1987; Savio et al., 1993a; Stock and Fortune, 1993). Induction of a low P_4 environment leads to the development of an estrogen active-persistent DF and has been used as a model for the study of

dominance in cattle (Savio et al., 1993a). Injection of $PGF_{2\alpha}$ and insertion of a Norgestomet implant (Savio et al., 1993a) or a CIDR-B (Controlled Internal Drug Release-Bovine) device on day 8 of the estrous cycle (Savio et al., 1993b), or injection of $PGF_{2\alpha}$ and insertion of a PRID (P_4 releasing intravaginal device) on day 5 of the estrous cycle (Wehrman et al., 1996) reduces concentrations of plasma P_4 and leads to development of a first wave persistent DF (FWPDF). Wehrman et al. (1996) reported that FSH treatment initiated 5 days after insertion of a 0.5 PRID reduces the number of corpora lutea (CL), total ova and transferable embryos. However, the ability of a persistent DF to suppress the response to exogenous FSH treatment depended upon time the DF was on the ovary, requiring more than 2 days to be able to exert dominance and this suppressive ability was lost after 9 days of treatment. Presence of a FWPDF is associated with a large number of small (≤ 5 mm) and very few medium size (5 to 9 mm) follicles which is indicative of a block in follicle recruitment and selection.

The primary objective of the present study was to evaluate the effect of FSH-P on superinduction of follicles and ovulatory responses in the presence or absence of an active-FWPDF that is known to inhibit spontaneous follicle recruitment.

Materials and Methods

Experiment 1

An initial experiment was conducted to evaluate the effect of removal, via aspiration, of an active and persistent first wave DF on folliculogenesis in response

to FSH superstimulation protocol A total of eight cyclic dairy cows was synchronized with an injection of GnRH (Buserelin 8 µg i m Receptal® Hoechst Roussel AgriVet Sommerville NJ) followed 7 days later by an injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ 25 mg i.m Lutalyse Pharmacia & Upjohn Kalamazoo MI Figure 4-1) Brightly colored enamel-based paint (Impervo Benjamin Moore Montvale NJ) was applied to the tailhead of each cow one day before injection of $PGF_{2\alpha}$

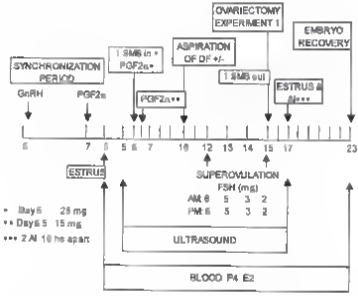

Figure 4-1 Experimental sequence for Experiment 1 and Experiment 2

On day of $PGF_{2\alpha}$ injection the paint strip was covered with a contrasting color of chalk (All-Weather® Paintstick® Lake Chem Co Chicago IL Van Cleeff et al 1986) The extent of tail paint and chalk (TPC) removal was scored based on a modification of the method of Macmillen et al (1985) using a scale of 5 (full

presence of paint and chalk not in estrus) to 0 (absence of paint and chalk, standing estrus Van Cleeff et al 1996) Scoring of TPC and visual detection of estrus were performed twice daily (0600 to 0700 h and 1800 to 1900 h) for 3 days after injection of $PGF_{2\alpha}$

On day 6 of the estrous cycle (day 0 = estrus) all cows received a 6 mg Norgestomet ear implant without injection of the norgestomet/estradiol valerate solution (Syncromate-B Sanofi Animal Health Inc Overland Park KS) and two injections of $PGF_{2\alpha}$ (25 mg AM and 15 mg PM i m) to induce luteolysis and create a low progestin environment for development of an active-FWPDF (Savio et al 1993b) The implant was left in place for 9 days Cows were assigned randomly to either a nonaspiration group (control group, n=4) or the treatment group in which the induced active-FWPDF was removed by transvaginal aspiration (treatment group, n=4)

The first wave DF was removed via transvaginal ultrasound guided aspiration on day 10 (e g 4 days after insertion of Norgestomet implant) utilizing an Aloka Echo-Camera SSD-500 unit (Aloka Co LTD Japan) equipped with a 5 0 MHz transvaginal convex array transducer and needle guide Epidural anesthesia was induced with 5 ml of 2% lidocaine and the perineal region was scrubbed and disinfected The lubricated transducer with needle guide was inserted deep into the vagina A 17-gauge 60 cm, single channel sterile needle fitted with a 19-gauge, 2 5 cm disposable tip was used in the needle guide to puncture the vaginal wall and peritoneum By manipulation of the reproductive tract per rectum ovaries were positioned and held firmly in front of the transducer in close apposition to the vaginal

wall. After aligning the on-screen needle travel indicator line with the follicle, the needle was advanced through the wall of the vagina, into ovarian stroma and through the wall of the follicle. As soon as the tip of the needle was seen to enter the follicle, follicular contents were aspirated into a sterile plastic tube using a suction unit (Pioneer Medical, Inc., Madison, CT) preset to a flow rate of 20 ml/min at a preset pressure of 100 mm Hg. Collection of follicular fluid into tube and collapse of the DF on screen were regarded as a successful aspiration of the follicle.

On the basis that an immediate rise in concentrations of FSH occurs after cautenzation of the DF (Adams et al., 1992b) or after removal of the ovary bearing the DF in which an increase in FSH precedes recruitment of a new follicular wave by 2 days (Badinga et al., 1992), follicular superinduction was initiated 2 days after aspiration of the active-FWPDF. For follicular superinduction, a total of 32 mg Armour units of FSH-P (FSH-P™, Schering-Plough, Animal Health, Kenilworth, NJ) was given per animal, with injections initiated on day 12 of the experimental estrous cycle (e.g., 2 days after follicular aspiration) at 0700 h and continued at 12 h intervals in a decreasing regime over a 4-day period (A.M./P.M. = 6/6, 5/5, 3/3 and 2/2 mg per injection). A real time ultrasound scanner (Aloka, Echo-Camera SSD-500) equipped with a 7.5 MHz transrectal linear array transducer was used to monitor size and number of ovarian follicles ≥ 2 mm in diameter from day 5 until overiectomy on day 15 of the experimental estrous cycle. Ovarian maps with the relative position of follicles and corpora lutea were drawn during each examination. According to the diameter, follicles were grouped into the following size classes

Class 1 ≤ 5 mm Class 2 6-9 mm Class 3 ≥ 10 mm Accumulated follicle diameter was obtained by summing the diameter of all follicle sizes within a class

Experiment 2

The objective was to evaluate folliculogenesis ovulatory responses and embryo yields to a superovulatory protocol with FSH-P administered in the presence or absence of an active-FWPDF Eleven cycling dairy cows were used in the experiment. Synchronization of estrus induction of an active-FWPDF follicle aspiration and induction of superovulation procedures were the same as in experiment 1 (Figure 4-1) However ultrasonography was extended until detection of estrus on day 17 Ultrasonography was used to confirm ovulation 2 days after estrus and to count number of corpora lutea on day of embryo recovery

Cows were inseminated twice at 12-h intervals starting 10 hours after detection of estrus Estrus detection was done in the same manner as described in experiment 1 Embryos were recovered nonsurgically on day 6 after insemination following a procedure described by Putney et al (1988) At the time of embryo collection number of embryos retrieved per cow was recorded and the embryos were classified by stage of development and quality according to the criteria of the International Embryo Transfer Society (1990) Embryos were grouped into six stages unfertilized embryos (UFO) 2-12 cell early morula morula early blastocyst and blastocyst Embryos were given a quality classification score of 1= excellent perfect embryo 2= good trivial imperfections 3= fair definite but no severe problems such as extruded cells or a small amount of degeneration and 4=

poor partly degenerated or vesiculated cells (Putney et al 1988) Potential embryos were classified as UFO plus all embryos Freezable and transferable embryos included only quality 1 and 2 embryos that were in the morula or blastocyst stages of development

Blood samples (10 ml) were collected daily from a tail vessel into heparinized tubes from day 0 (first estrus) until the day of ovariectomy (experiment 1) or until the day of embryo recovery (day 6 of next cycle, experiment 2) Blood samples were stored in an ice bath and plasma separated by centrifugation (1 800 *g* for 15 min) and stored at -20°C until assayed for P_4 and estradiol (E_2) Concentrations of P_4 were measured by radioimmunoassay (RIA Knickerbocker et al 1986) in all samples Sensitivity of the P_4 assay was 0 28 ng/ml Intra- and inter-assay coefficients were 8 5% and 13 1% respectively Concentrations of E_2 in plasma from day 0 to day 15 (day of ovariectomy, experiment 1) or until day of estrus (experiment 2) were measured by RIA as described by Tortonese et al (1990) and modified by Badinga et al (1992) Sensitivity of the assay was 0 8 pg/ml and the intra- and inter-assay coefficients of variation were 8 5% and 22 6% respectively

Statistical Analyses

Data were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS 1988) Data from both experiments were analyzed together since inclusion of replicate in the mathematical model was not significant. The mathematical model used to analyze the number of follicles and the accumulated follicle diameter within discrete size

classes size of the dominant follicle and plasma concentrations of P_4 and E_2 included effects of treatment cow(treatment) day of the cycle and treatment by day interaction The main effect of treatment was tested using the mean square of cow(treatment) as the error term When interaction treatment by day was significant, data were analyzed using orthogonal contrasts to compare hormonal concentration between groups Plasma concentrations of E_2 were analyzed in two periods from day 0 to day 10 and from day 10 to day 16. Plasma concentrations of P_4 were analyzed from day 0 to day of embryo recovery To correct for heterogeneity of variance plasma concentrations of P_4 were analyzed after logarithmic transformation (natural log) of P_4 concentration + 5 from day 16 to day of embryo recovery

A one way analysis of variance was used to analyze discrete responses to superovulation treatment such as follicles > 9 mm on the day of estrus number of ovulatory follicles (difference between the number of follicles > 9 mm present in the ovary on the day of estrus and the number of nonovulatory follicles 2 days after ovulation) percentage of ovulatory follicles (number of ovulatory follicles as a percentage of follicles > 9 mm present in the ovary on the day of estrus) number of nonovulatory follicles (number of follicles > 9 mm remaining in the ovary two days after ovulation), number of corpora lutea (number of CL present in the ovary the day of embryo recovery as determined by ultrasonography), number of UFO and embryos (sum of UFO plus all embryos) recovery rate relative to ovulatory follicles (rate between number of UFO plus embryos and number of ovulatory follicles) recovery rate relative to number of CL (number of UFO plus embryos as a

percentage of CL number) number of embryos fertilization rate (ratio between number of embryos and total number of UFO and embryos) number of transferable embryos percentage of transferable embryos relative to number of UFO and embryos (number of transferable embryos as a percentage of total number of UFO and embryos) percentage of transferable embryos relative to number of embryos (number of transferable embryos as a percentage of embryos) percentage of embryos relative to number of CL (number of embryos as a percentage of total CL number) and percentage of transferable embryos relative to number of CL (number of transferable embryos as a percentage of total CL number)

Results

Follicular Dynamics

The first wave dominant follicle was identified in all cows at the ultrasound examination on day 5 of the cycle Under a low progesterone environment created by insertion of the norgestomet ear implant and $PGF_{2\alpha}$ injections on day 6 the FWPDF increased its size from 11 0 ± 0 6 mm on day 5 to 15 8 ± 0 6 mm on day 10 in the control group and from 10 3 ± 0 6 mm on day 5 to 15 1 ± 0 6 mm on day 10 for the treatment group (Figure 4-2) In the control group the FWPDF continued growing and reached 19 9 ± 0 7 mm on day 16 of estrous cycle Follicular aspiration in the treatment group was associated with disappearance of the active-FWPDF

Number of class 1 follicles (Figure 4-3A) did not differ between groups from

day 5 until day 10 and from day 15 until day 17 Number and accumulated diameter (Figure 4-3B) for class 1 follicles decreased ($P < 0.05$) in the treatment group from day 11 until day 15 Number of class 1 follicles on day 10 (34.1 ± 3.1 Figure 4 3A) decreased to 3.2 ± 3.9 on day 16 and accumulated class 1 follicle diameter decreased from 94.9 ± 9.4 mm on day 10 to 13.8 ± 12.1 mm on day 16 (Figure 4-3B) In contrast, for the control group number of class 1 follicles increased from day 10 (30.7 ± 2.9) until day 12 (41.4 ± 3.1) and accumulated class 1 follicle diameter increased from day 10 (97.4 ± 6.9 mm) until day 13 (126.5 ± 9.5 mm Figure 4-3B) before decreasing to the same levels at day 16

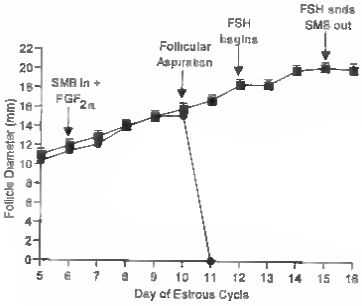

Figure 4-2 Pattern of growth of the first wave dominant follicle from day 5 to day 16 of the estrous cycle in control (■) and treatment (●) cows

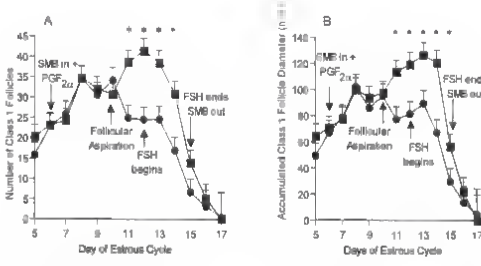

Figure 4-3 Number (LSM ± SE, A) and accumulated follicle diameter (mm LSM ± SE B) for class 1 follicles from day 5 to day 17 in control (■) and treatment (●) cows Asterisk (*) indicates differences ($P < .001$) between means within day

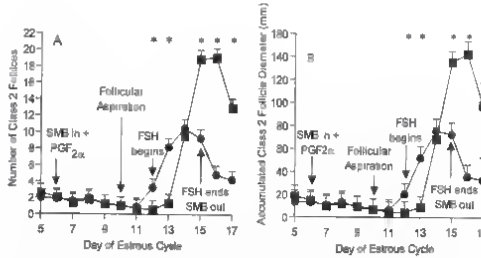

Figure 4-4 Number (LSM ± SE, A) and accumulated follicle diameter (mm LSM ± SE B) for class 2 follicles from day 5 to day 17 in control (■) and treatment (●) cows Asterisk (*) indicates differences ($P < .001$) between means within day

The immediate reduction in class 1 follicles in the treatment group, was associated with increased recruitment (3 3 > 0 6 ± 1 2 P < 0 1) and accumulated follicle diameter (20 5 > 4.2 ± 8 6 mm P < 0 1) of class 2 follicles (Figure 4-4 A and B) on day 12 just prior to FSH-P injections and on day 13 (1 day after FSH-P injections began) to 6 2 > 1 4 ± 1 2 and 52 0 > 9 1 ± 9 2 mm for number and accumulated diameter of class 2 follicles respectively (P < 0 001) The decline in number of class 1 follicles of the control group (Figure 4-3A) did not occur until day 14 (30 9 ± 3 1) at which time there was an increase in number of class 2 follicles (9 6 ± 1 3 Figure 4-4A) This increase continued until day 15 for the control group (16 9 ± 1 3) when number of class 2 follicles was significantly less and declining in the treatment group (9 3 ± 1 4 P < 0 001 Figure 4-4A) Accumulated diameter of class 2 follicles followed the same pattern as number of class 2 follicles (Figure 4-4B)

The decline in number of class 2 follicles of the treatment group was associated with an earlier increase and greater numbers of class 3 follicles on days 14 (5 9 ± 1 2 vs 1 0 ± 1 1 P < 0.001 Figure 4-5A) 15 (15 9 ± 1.2 vs 5 8 ± 1 1 P < 0 001 Figure 4-5A) and 16 (15 6 ± 1 5 vs 9 7 ± 1 3 P < 0 001) compared to the control group Presence of an active FWPDF delayed the FSH-induced increase in class 3 follicles by 1 to 2 days Number of class 3 follicles was not different significantly between groups at day 17 (23 7 ± 2 3 vs 19 6 ± 2.3) However accumulated diameter of class 3 follicles was significantly greater in the treatment group (319 0 ± 29 5 mm vs 220 0 ± 29.5 mm P < 0 01 Figure 4-5B) due to an earlier appearence in class 3 follicles on the ovary contributing to a greater

accumulated diameter These differences in dynamics of follicle change reflect the inhibitory effect of the active-FWPDF that can be over-ridden with continuous treatment of exogenous FSH-P

Plasma Progesterone and Estradiol 17β Concentrations

Concentrations of P_4 in plasma did not differ between the two groups (Figure 4-6A) Concentrations of plasma P_4 increased from day 1 (0 6 ± 0 6 ng/ml) until day 6 (5 6 ± 1 9 ng/ml) at which time two injections of $PGF_{2\alpha}$ were administered After injection of $PGF_{2\alpha}$, plasma concentrations of P_4 decreased to basal levels and remained low (< 1 ng/ml) until day 19 when they increased to 3 0 ± 3.5 ng/ml in the control group and 4 7 ± 3 8 ng/ml in the treatment group Day 19 was approximately 1 to 2 days after ovulation On the day of embryo recovery plasma concentrations of P_4 were the same for both groups The tendency of higher P_4 concentrations in the control group is due to one cow which had 10 8 4 ng/ml of P_4 and 19 CL

Concentrations of plasma E_2 were higher ($P < .001$) in the control group than in the treatment group on day 8 (8 1 ± 0 7 pg/ml vs 6 1 ± 0 8 pg/ml) day 9 (9 6 ± 0 7 pg/ml vs 5 9 ± 0 8 pg/ml) day 10 (11 5 ± 0 7 pg/ml vs 5 9 ± 0 7 pg/ml) and after follicular aspiration on day 11 (13 5 ± 0 6 pg/ml vs 2 7 ± 0 9 pg/ml), day 12 (14 8 ± 0 8 pg/ml vs 3 1 ± 0 9 pg/ml), and day 13 (17 1 ± 0 9 pg/ml vs 7 1 ± 0 9 pg/ml Figure 4-6B) of the oestrous cycle

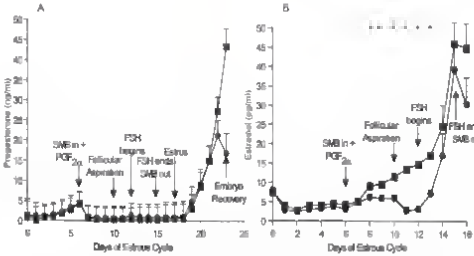

Figure 4-6 Concentrations of progesterone (LSM ± SE A) from day of estrus (day 0) to day of embryo recovery and estradiol-17β (LSM ± SE B) from day of estrus until day 18 of the estrous cycle in control (■) and treatment (●) cows Asterisk (*) indicates differences ($P < .001$) between means within day

Ovarian Responses to Superovulation Treatment

On the day of detected estrus number of follicles > 9 mm number ofovulatory follicles percentage of ovulatory follicles and number of nonovulatory follicles did not differ between treatments (Table 4-1) The array of other ovarian and embryo responses (Table 4-2) measured on the day of embryo recovery did not differ between groups

Table 4-1 Ovulatory responses (mean ± SEM) to a superovulatory treatment measured the day of detected estrus

	Follicular Aspiration	
	+ (Treatment)	- (Control)
Number of animals	5	6
Total of follicles > 9 mm	16.4 ± 4 4	13 0 ± 3.9
Number of ovulatory follicles	12.0 ± 5 4	7 2 ± 4 9

Table 4-1 continued

	Follicular Aspiration	
	+ (Treatment)	(Control)
Ovulatory follicles (%)	58 7	50 7
Number of nonovulatory follicles	6 4 ± 3 2	5 6 ± 2 9

Table 4-2 Ovulatory response (mean ± SEM) to superovulatory treatment measured the day of embryo recovery

	Follicular Aspiration	
	+ (Treatment)	(Control)
Number of animals	5	6
Number of corpora lutea (US)*	10.2 ± 4 6	7 5 ± 4 2
Number of ova & embryos	5 0 ± 3.6	6.2 ± 3.6
Recovery rate of ova & embryos/# of CL (%)	35.2	44 2
Recovery rate of ova & embryos/# ovulatory follicles (%)	32 6	46 1
Number of embryos	2.6 ± 1 6	2 7 ± 1 7
Fertilization rate (%)	60 4	34 5
Number of transferable embryos	1 0 ± 1 6	2.3 ± 1 5
Transferable embryos/total number of ova & embryos (%)	43 3	16 6
Transferable embryos/total number of embryos (%)	50 0	35 1
Number of embryos/number of CL (%)	24 6	20 6
Transferable embryos/number of CL (%)	14 6	3 4

* US= ultrasound

Discussion

Growth of the first wave DF is one of the most predictable events in ovarian follicular dynamics during the estrous cycle in cattle (Badinga et al. 1992, Fortune, 1993). Demise of the first wave DF at midcycle is due to the negative feedback effect of P_4 from the corpus luteum on LH secretion (Savio et al. 1993a, Kinder et

al 1995). LH secretion during the estrous cycle in cattle is characterized by LH pulses of high frequency and low amplitude during proestrus and metestrus and low frequency, high amplitude pulses during diestrus (Rahe et al. 1980). Progesterone and E_2 play an important role in regulation of pituitary LH secretion. Under a low progestin treatment in the absence of a CL, the pattern of LH secretion is similar to that during the follicular phase of the estrous cycle in the cow (Kindar et al. 1996). In such a low progestin environment and an increased LH pulse frequency, the first wave DF continues to grow and suppresses growth of other follicles (Savio et al. 1993a, 1993b). In the present study, induction of a low progestin environment resulted in persistence and increased size of the DF from days 6 to 16, which is in agreement with others (Sirois and Fortune, 1990; Savio et al., 1993b). Prolonging the period of follicular dominance is an alternative model to study whether a DF can alter induced follicular development in response to exogenous FSH.

The phenomenon of dominance is central to understanding folliculogenesis since it suggests that some follicles survive in a milieu suppressive to growth of other follicles. In addition, dominance is related to the success of superovulation both in humans and domestic animals (Fortune, 1993). There are numerous reports of superovulation programs in cattle with variable results (Elsden et al., 1979; Murphy et al., 1984; McGowan et al., 1985; Lerner et al., 1986; Pawlyshyn et al., 1986). The ovarian response to a superovulation program is determined by many factors such as ovarian responsiveness of donors, fertilization rate, embryo viability, factors related to physiological status of the animal such as pregnancy, and

management (convenience and cost effectiveness of superovulation protocols Armstrong 1993)

Ovarian status at the time of superovulatory treatment has been postulated as a major factor determining ovarian response (Monniaux et al 1983) The increase in number of class 2 follicles at day 2 after aspiration of the active-FWPDF of the present study indicates that the FWPDF suppressed growth of follicles This suppressive influence may have been through secretion of some factors such as estradiol and/or inhibin, that deprived follicles of gonadotropin support critical for their further development (Fortune 1993) Recruitment was attenuated in the presence of the active-FWPDF as shown by a lower number of class 2 follicles in the control group until day 14 which was 2 days after initiation of FSH treatment. Similar results were reported by Guilbault et al (1991) when they superovulated heifers in the presence of a DF At day 14 recruitment was evident in the control group as the number of class 2 follicles increased in association with a decrease in number of class 1 follicles This process occurred 2 days earlier in the treatment group (day 12) in which the active-FWPDF was aspirated on day 10 Likewise number of class 3 follicles increased earlier in the treatment group without an active-FWPDF However by day 17 the number of class 3 follicles was the same for the two groups indicating that exogenous FSH eventually overrides the inhibitory influence of the active-FWPDF on the ovaries of the control group Nevertheless accumulated follicle diameter was higher for the aspirated group than for the control group with the active-FWPDF This is due likely to earlier occurrence and longer period for growth in the treatment group

Initiation of the superovulatory regime in the absence of a DF has been shown to improve the ovarian response to FSH (Grasso et al 1989 Guilbault et al 1991 Savio et al 1991 Huhtinen et al 1992 Bungartz and Niemann 1994 Wolfsdorf et al 1997) However Gray et al (1992) found no difference in yield of transferable or total embryos when FSH treatment began during the period of morphological regression of the DF versus a control group in which superovulatory treatment began on day 10 when DF was morphologically dominant Ovulation rate decreased when FSH was given in the presence of a DF resulting in fewer embryos recovered when compared with initiation of treatment when a DF is not present (Guilbault et al 1991 Huhtinen et al 1992) This beneficial effect was not shown in the present experiment where none of the variables measured on the day of estrus or the day of embryo recovery were different Likewise follicular development ovulation rate and embryo recovery rate did not differ when first wave DF was ovulated with hCG and superovulation treatment started in the absence of a DF (Rajamahendran and Calder 1993) Stock et al (1993) reported no differences in number of ovulatory size follicles ovulation rates or yield of total or transferable embryos between heifers superovulated in the absence or the presence of a DF on day 6 However in this latter experiment 8 of the 10 animals in the group with the DF present ovulated during FSH treatment. In the present experiment the active-FWPDF was present in the ovary of all control cows during FSH-P treatment and all FWPDFs ovulated after estrus In both the present study and that of Wehrman et al (1996) presence and duration of a DF were controlled experimentally to evaluate effects of a DF during FSH treatment.

Transvaginal aspiration of the active-FWPDF demonstrated that factors from the follicle exert inhibitory effects on follicular growth at the ovarian level Immediately upon aspiration of the active FWPDF changes in number of class 1 and class 2 follicles occurred that were indicative of follicular recruitment prior to initiation of FSH treatment Presence of an active-FWPDF during FSH-P treatment decreased the response to exogenous FSH-P during the first days of treatment compared to the treatment group with no FWPDF With continuous administration (4 days) of FSH-P the inhibitory effect of the active-FWPDF was overridden resulting in the same number of class 3 follicles at estrus as well as the same ovulatory and embryo responses Wehrman et al (1996) reported a reduced number of CL total ova and transferable embryos when the FSH treatment was given 5 days after insertion of a 0 5 PRID but not when FSH was started 2 or 3 days after insertion In the present experiment the norgestomet implant was inserted on day 6 and FSH treatment started 4 days later

It has been shown that passive immunization of ewes against steroid free-follicular fluid or against synthetic inhibin peptides increased ovulation rates two- to four-fold (O'Shea et al 1991 Schanbacher et al 1991) Ovulation rate and prolificacy in the cow are more difficult to manipulate by inhibin vaccination (O'Shea et al , 1994) However there are reports that immunization of heifers or cows against steroid-free follicular fluid increases ovulation rate (Price et al., 1997 Glencross et al 1992 Morris et al 1993, Scanton et al ., 1993) and number of transferable embryos (Alvarez et al ., 1997) Thus immunoneutralization of inhibin increases ovarian follicular development

Follicular dominance appears to be controlled by a number of mechanisms acting in concert. These include alterations in peripheral FSH concentrations in response to E_2 and inhibin secreted by the DF as well as the possible production of local ovarian factors that inhibit development of subordinate follicles (Campbell et al 1995 Armstrong and Webb 1997). Two hypotheses have been advanced to explain how the DF exerts dominance. The DF could cause regression of subordinate follicles indirectly via a negative feedback on FSH secretion (Fortune 1994). Alternatively the DF may secrete a factor that directly impairs further growth and development of subordinate follicles (Ko et al 1991 Adams et al 1992a Fortune 1994). In monotocous species such a factor would clearly have to be endocrine in nature since it would need to inhibit recruitment and induce regression of subordinate follicles on both ovaries. Besides the peripheral action of inhibin on FSH secretion there is strong evidence supporting regulatory actions of inhibin-like molecules within the ovary (Hsueh et al 1987 Hillier et al 1991a 1991b Woodruff et al 1990 Schneyer et al 1991). Sato et al (1992) suggested that an inhibin-like substance inhibits FSH action at the ovarian level through binding to the FSH receptor on the granulosa cell. More recently a report by Schneyer et al (1991) indicated that inhibin α-subunits bind to FSH receptor sites and inhibit FSH bioactivity in granulosa cell cultures.

Neither number of CL nor plasma P_4 on the day of embryo recovery differed between groups. Higher concentrations of E_2 in plasma during days 8 9 and 10 of the estrous cycle before aspiration of the FWPDF were due to variability among cows of the control group being greater than cows of the treatment group prior to

aspiration of the FWPDF on those days ($P < 0.01$). This was due to eight of nine cows in the control group having an increase in plasma E_2 of > 5 pg/ml whereas only five of eight cows in the treatment group had this increase. On days 11, 12, and 13, the difference between treatments in plasma E_2 was due to presence of an active-FWPDF in the control group. Larger class 3 follicles reflected by a higher accumulated follicle diameter for this follicle class in the treatment group on days 16 and 17 was not associated with higher concentrations of E_2 in plasma.

Implications

Initiation of a superovulation treatment in the absence of an active-FWPDF increased the number of class 2 follicles (6 to 9 mm) present on the ovary one day after beginning FSH-P injections. This response was associated with an increase in number of class 3 follicles (≥ 10 mm) on the last day of FSH-P injection. Increased follicle recruitment following removal of the FWPDF is an indication that presence of an active-FWPDF had an inhibitory effect on follicular responses to exogenous FSH. However, this enhanced ovarian stimulatory effect was not present at the time of estrus or embryo collection. Thus inhibition exerted at the ovarian level appears to be over-ridden by continuous exogenous exposure to FSH-P.

Additional research is required to investigate the effects of inhibin or inhibin forms present in follicular fluid of dominant follicles on granulosa cells *in vitro* to elucidate the role of inhibins at the local level within the follicle. To study these effects, it is necessary to develop an estrogenic and FSH responsive test system

comprised of bovine granulosa cells in culture. Development of such a system is the focus of Chapter 5.

CHAPTER 5
IN VITRO SECRETION OF ESTRADIOL BY BOVINE ANTRAL GRANULOSA CELLS

Introduction

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are essential for development and maintenance of granulosa (GC) and theca cells (TC) in ovarian follicles in mammals. Secretion of FSH and LH is regulated in a positive manner by gonadotropin releasing hormone or inhibited by ovarian hormones such as steroids and inhibin (Taya et al. 1998). Ovarian follicle GC are epithelial cells that produce E_2 upon proliferation account for the majority of follicle cell expansion and support the developing oocyte (Skinner and Coffey 1988). Theca cells are mesenchymal cells that surround the follicle which contribute to its expansion and also produce steroid hormones such as androgens (Skinner and Coffey 1988).

Granulosa cells have an integral role in the maintenance and control of ovarian function through the biosynthesis of estrogens and progesterone. Therefore regulation of GC function influences both local ovarian function and the endocrine status of the female (Skinner and Osteen 1988). In most mammalian species TC synthesize androgens from progestins but lack aromatase activity whereas GC cannot convert progestins to androgens but can aromatize androgens to estrogens (Berndtson et al. 1995). In the bovine follicle the major steroid

product of GC is pregnenolone (Greenwald and Roy 1994) which is apparently exported to the TC for ultimate conversion to androgens (Fortune 1986 Fortune and Quirk 1988 Roberts and Skinner 1990) and the androgens are utilized by the GC to produce E_2 (Fortune 1986 Fortune and Quirk 1988 Roberts and Skinner 1990) Results from Fortune (1988) and Roberts and Skinner (1990) support a potential role for E_2 produced by GC in the enhancement of thecal pregnenolone and androstenedione production but inhibits P_4 secretion by inhibiting the conversion of pregnenolone to P_4 by 3ß-hydroxysteroid dehydrogenase (Fortune 1986) Estradiol thereby increases the use of the Δ^5 pathway (Fortune 1986)

A large variety of membranous and intracellular receptors have been identified in GC at different stages of differentiation For example peptide hormones neurotransmitters steroid hormones growth factors and extracellular matrix components interact in a complex manner to control biochemical and structural differentiation of GC (Amsterdam and Rotmensch 1987) Cultures of mammalian GC have been demonstrated by several investigators (rat: Dorrington and Armstrong 1979 pig Baranao and Hammond 1985 Bareño and Hammond 1985 Hyika et al 1989 and human Schipper et al 1993 sheep Campbell et al 1996 cattle Saumande 1991 Spicer and Alpizar 1994 Berndtson et al 1995 Rouillier et al 1996 Gutiérrez et al 1997)

Reports regarding effects of FSH on bovine GC cultured *in vitro* are controversial with results depending upon type of GC cultured (antral versus mural GC) conditioned medium utilized (serum-free media versus media containing fetal calf serum) dose of FSH utilized for cell stimulation (low vs high doses) type of

follicle (small vs large) as a source of GC treatment used to obtain the follicles (use of FSH-P vs PMSG as stimulatory hormones for follicular growth) and time in culture (short term vs long term cultures). Saumande (1991) reported that low doses of FSH (2 ng/ml) were able to stimulate the production of E_2 by bovine antral GC in a long term culture with a defined serum-free medium. Bamdtson et al (1995) using serum-free and serum-containing media showed that mural GC were able to respond to very low doses of FSH (1 and 2 ng/ml) and stimulate E_2 production whereas higher doses (8 - 128 ng/ml) accentuated the natural tendency of GC to luteinize *in vitro* and secrete progesterone (P_4). Routtiar et al (1995) demonstrated that mural and antral bovine GC cultured in a completely defined medium responded morphologically and functionally to FSH in a long-term culture. However FSH- and LH-induced production of E_2 and P_4 productions was considerably lower in mural than in antral bovine GC. It has been demonstrated that FSH (Kuran et al 1995) LH and equine chononic gonadotropin (eCG Kuran et al 1996) stimulated differentiation and P_4 production by mural (Kuren et al 1995) and antral GC (Kuran et al 1996) cultured in serum-free medium. More recently Gutiérrez et al (1997) showed that a serum-free culture system is suitable for mural GC culture. Production of E_2 can be induced and/or maintained with cells remaining responsive to FSH at physiological concentrations to increase proliferation and estrogen secretion. The objective of this experiment was to study the effect of low doses of FSH on induction of E_2 secretion by antral bovine GC in long term culture.

Materials and Methods

Granulosa Cell Isolation

Cycling Holstein cows (n=3) were synchronized with a GnRH injection (Buserelin 8 µg i.m. Receptal® Hoechst-Roussel Agri Vet, Sommerville NJ) followed 7 days later with an injection of prostaglandin (PG) F_2alpha (25 mg i.m. Lutalyse, Pharmacia & Upjohn Co., Kalamazoo MI. Figure 5-1)

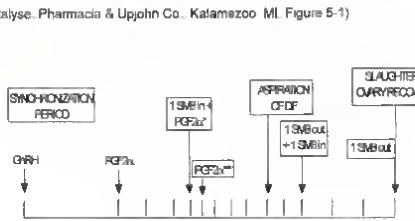

Figure 5.1 Experimental sequence followed to obtain superstimulated ovaries to harvest bovine antral granulosa cells for culture

On day 6 after estrus (day 0= day of estrus) cows received a Norgestomet ear implant (6 mg norgestomet implant without the injection of the norgestomet/valerate solution Syncromate Sanofi Animal Health, Inc. Overland Park KS) and were given two injections of PGF_2alpha (25 mg i.m. AM and 15 mg

i m., PM) to create a low progestin environment for the development of a persistent dominant follicle (Savio et al 1993) The implant was left in place for 6 days and then replaced by a new implant on day 12 when FSH treatment was initiated On day 10 the persistent dominant follicle was aspirated via transvaginal ultrasound guided aspiration using the Aloka Echo-Camera SSD-500 (Aloka Co LTD Japan) equipped with a 5 0 MHz transvaginal convex array transducer and aspiration needle guide Two days later on day 12 a treatment of FSH (FSH-P™ Schering-Plough Animal Health Kenilworth NJ) was initiated Twenty eight mg were given i m at 12 h intervals in a decreasing regime over a 3-day period (a m./p m = 6/6 5/5 and 3/3 mg) On day 15 cows were slaughtered ovaries were collected and placed immediately in sterile saline solution (9% NaCl at 37°C) containing streptomycin (2 mg/ml) and fungizone (0 1 mg/ml) and transported to the laboratory The rationale for the use of this follicle induction system is as follows with creation of a persistent DF, growth of small follicles was suppressed after aspiration of the persistent DF or removal of the ovary bearing the DF there is an endogenous surge of FSH (Badinga et al 1992) that promotes recruitment of new follicles The FSH-P treatment was begun 2 days after aspiration of the DF to supplement the endogenous FSH recruitment of follicles and to stimulate growth to a desirable follicle size of ≥ 9 mm Ovaries were trimmed and washed twice with saline solution containing antibiotics and antimycotics Ovaries were then wiped off with gauze containing 70% ethanol and placed in saline solution until GC were harvested Antral GC were collected as described by Saumande (1991) except that Menezo B_2 medium (Laboratoire C C D France) was used instead of INRA F_1

medium to collect GC from follicles. Consecutive follicles ≥ 9 mm in diameter were punctured with a blunt 18-gauge needle attached to a 5-ml syringe and slowly rinsed 10 times with 0.5 ml of Menezo B_2 medium supplemented with heparin (100 IU/ml; Sigma, St. Louis, MO). These follicles were E_2-active based on follicular fluid concentrations (E_2 = 351 ng/ml). Ireland and Roche (1983) designated E_2 active follicles to have ≥ 141 ± 35 ng/ml in follicular fluid. Granulosa cell suspension was centrifuged (300 x *g* for 7 min), the pellet resuspended in 0.5 ml of Menezo B_2 medium supplemented with ethylene glycol tetraacetic acid (EGTA, 2.47 mg/ml; Sigma, St. Louis, MO) and maintained in an incubator for 15 min at 37°C. Following centrifugation (300 x *g* for 7 min), cells were resuspended in Menezo B_2 medium. Cell viability (62.6%) was determined by trypan blue exclusion (Sigma, St. Louis, MO; 4% final concentration) using a hemocytometer. Cell suspension was adjusted in Menezo B_2 medium at a concentration of $3x10^5$ cells in 50 µl.

Granulosa Cell Culture

Granulosa cells were cultured for 48, 72 or 96 h in 48-well culture plates (Costar, Cambridge, MA), with each one cm^2 well precoated with fibronectin (3 µg in 200 µl; Sigma, St. Louis, MO) for 30-60 min at 37°C in Ham's F-12 (Sigma, St. Louis, MO). Cells were plated at a density of $3x10^5$ cells/well (50 µl) and 250 µl of medium added to complete a 500 µl volume/well. Ham's F-12 was buffered with $NaHCO_3$ (1.176 mg/ml) and Hepes (20 mM; Sigma, St. Louis, MO), pH 7.4, and was supplemented with insulin (50 ng/ml; Sigma, St. Louis, MO), androstenedione (10^{-7} M; Steraloids, Inc., Wilton, NH), human transferrin (partially iron-loaded; 10 µg/ml;

Sigma St Louis MO) ascorbic acid (17 6 µg/ml Sigma St Louis MO) gentamicin (20 µg/ml Sigma St. Louis MO) nystatin (4 µg/ml Sigma St. Louis MO) and no FSH or 0 5 ng/ml of respective FSH's Medium was sterilized using a 0 22 µm filter (Naigene® Naige Nunc International Rochester NY) The following FSH's were evaluated 1) porcine FSH (prepared by Dr Y Combarnous [CY-FSH] INRA Nouzilly France, batch CY 1737 III) FSH activity 41 x NIH FSH P1 LH activity < 5%) 2) porcine FSH (prepared by Dr J F Beckers [B-FSH]. University of Liege Belgium FSH activity 10 µg of pure FSH correspond to 1 mg Armour unit or USDA S1 LH content < 1%), and 3) ovine FSH (USDA-oFSH-19 SIAFP-RP-2 prepared by Dr A. F Parlow [USDA-FSH] Pituitary Hormones and Antisera Center Torrance CA FSH activity 94 x NIH-oFSH-S1 LH activity 025 x NIH-oLH-S1 in 10 mg of BSA) The priming dose of 0 5 ng/ml was used in order to optimize the *in vitro* secretion of E_2 (Rouillier et al 1997) and in conjunction with Insulin to improve the maintenance of cell viability (Saumande 1991) Cells were cultured in a humidified incubator with an atmosphere of 5% CO_2 and 95% air at 37°C Culture medium was collected and replaced daily with 500 µl of fresh medium At 24 48 or 72 h of culture cells were treated with different concentrations of the respective FSHs 0 0 5, 1 2 4 6 8 10 and 50 ng/ml (3 replicates per dose of FSH)

Granulosa Cell Number

The number of viable cells at 48 72 and 96 h was measured using the supravital dye neutral red (Sigma St. Louis MO) which selectively targets lysosomes in living cells and detects cell viability (Borenfreund and Puerner 1984)

At the end of the culture period spent medium was replaced with 500 µl/well of culture medium containing 25 µg of neutral red and plates were then incubated for 3 h at 37°C After incubation the neutral red solution was removed from the wells by inversion and replaced with 500 µl/well of a solution of formol-calcium (4% formaldehyde 1% calcium chloride in distilled water) to remove unincorporated neutral red and enhance attachment of cells to the substratum After 3 min which includes addition of formol-calcium and centrifugation (300 x g) the formol-calcium was removed and 500 µl/well of acetic acid-ethanol solution (1 ml glacial acetic acid in 100 ml of 50% ethanol) added to the wells to extract the neutral red The plates were allowed to sit at room temperature for 15 min and then shaken briefly to distribute the dye evenly Absorbance of the solution was determined at 540 nm using a Beckman DU-20 Spectrophotometer (Beckman Instruments Inc Irvine CA) The readings were averaged and compared with a standard curve generated on the first day of culture For a standard curve 0 $5x10^4$ $1x10^5$ $2x10^5$ $3x10^5$ $4x10^5$ and $5x10^5$ cells (n= 3/cell concentration) were incubated for 3 h at 37°C in 12x75 mm borosilicate tubes in presence of 500 µl of neutral red dye At the end of the incubation tubes were centrifuged for 7 min at 300 x g supernatant poured off slowly and 500 µl of formol-calcium added During a 3 min period tubes were centrifuged again at 300 x g supernatant poured off slowly and 500 µl of acetic acid-ethanol added The interval between addition of formol-calcium and completion of centrifugation must be performed within a 3 min period Tubes were incubated at room temperature for 15 min briefly shaken and absorbance read

using a Beckman DU-20 Spectrophotometer The relationship between number of cells and absorbance was linear ($y = -.02348 + 3 \times 10^{-6} X$ where y= absorbance and X= number of cells) Regression equation was inverted ($\hat{y} = 0.782 \times 10^{4} + 33.33 \times 10^{-5} X$ where $\hat{y}$ = number of cells and X = absorbance $r^2 = .98$) and the number of cells per well in culture plates was estimated from the resulting linear regression equation

Radioimmunoassays

Concentrations of E_2 and P_4 in unextracted cultured medium were determined with assays validated as described below Unextracted media samples (100 µl) were assayed in duplicate for E_2 and P_4 Medium (100 µl) was added to standard curve tubes to mimic the conditions of sample tubes Estradiol-17ß standards were made by serial dilutions from stock solution of 1 3 5(10)-estratrien-3,17ß-diol (Steraloids Inc Wilton, NH) diluted in benzene Standard solutions were diluted in phosphate saline buffer with 1% of gelatin (PBSg) pH 7 5 at final concentrations of 0 039 0 078 0 156 0 312 0 625 1.25 2 5 and 5 ng/100 µl. A 100 µl aliquof of estradiol [6 7 ^{3}H(N)] (50 0 Ci/mmol DuPont NEN Wilmington DE) at approximately 65 000 dpm diluted in PBSg was added to all tubes Antiserum at 1:20,000 dilution (100 µl, Gift from Dr Lars Edqvist,Uppsala Sweden) was added to give a final volume of 400 µl After overnight incubation at 4°C free and bound fractions were separated by 500 µl of a charcoal-dextran solution (250 mg/25 mg in 100 ml of PBSg 15 min incubation) Following centrifugation (15 min) at 2 618 x g supernatant was poured into scintillation vials and radioactivity counted Mean

(± SEM) concentrations of E_2 in 50 and 100 µl of a pooled sample diluted 1:5, 1:10 and non diluted were 29.9 ± 1.3, 29.3 ± 1.3, 29.1 ± 1.3 ng/ml for 50 µl of the pool and 27.1 ± 1.3, 26.8 ± 1.3 and 24.6 ± 1.3 ng/ml for 100 µl of the pool, respectively. Differences in concentrations of E_2 (ng/ml) because of sample volume and dilution were not detected. The sensitivity of the assay was 0.39 ng/ml. Intra- and inter assay coefficients of variation were 9.9% and 4.9%, respectively.

Progesterone standards were made by serial dilutions from a stock solution of 4-pregnan-3,20-dione (Steraloids, Inc., Wilton, NH) diluted in benzene. Standard solutions were diluted in phosphate saline buffer with 1% of gelatin, pH 7.5 at final concentrations of 0.31, 0.62, 1.25, 2.5, 5, 10, 15, 20 and 25 ng/100 µl. A 100 µl aliquot of [1α, 2α(n)-^{3}H]Progesterone (49.0 Ci/mmol, Amersham International plc, Arlington Heights, IL) at approximately 75 000 dpm diluted in PBSg, pH 7.5 was added to all tubes. Antiserum at a 1:15 000 dilution (100 µl, Gift from Dr. Magaly Manzo, Universidad Central de Venezuela, Knickerbocker et al., 1986) and 300 µl of PBSg were added to give a final volume of 700 µl. After overnight incubation at 4 °C, free and bound fractions were separated by 500 µl of a charcoal-dextran (500 mg/50 mg in 100 ml of PBSg, 15 min incubation) solution. Following centrifugation (15 min) at 2 619 x *g*, supernatant was poured into scintillation vials and counted. Mean (± SEM) concentrations of P_4 in 25, 50 and 100 µl of a pooled sample diluted 1:5, 1:10 and non diluted were 95.3 ± 5.5, 101.1 ± 5.5 and 94.0 ± 5.5 ng/ml for 25 µl of pooled sample, 117.9 ± 6.1, 105.2 ± 5.5, and 104.1 ± 5.5 ng/ml in 50 µl of pooled sample, 117.5 ± 5.5, 104.8 ± 5.5 and 95.3 ± 5.5 ng/ml in 100 µl of a pooled sample, respectively. Differences in concentrations of P_4 due to sample volume and

dilution were not detected Sensitivity of the assay was 3 12 ng/ml Intra- and inter-assay coefficients of variation were 3 9% and 10 8% respectively

Gel Electrophoresis

Samples of the different FSH types (CY FSH 600, 700 and 800 ng/gel lane B-FSH 600 ng/gel lane and USDA-FSH 500 ng/gel lane) were mixed in sample loading buffer containing 4% of sodium dodecylsulphate (SDS 6DH Laboratory Supplies Poole England) without (nonreducing conditions) β-mercaptoethanol boiled for 5 min and subjected to one dimensional 15% SDS-PAGE (Laemmli 1970) using a Bio-Rad mini gel apparatus (Mini-Protean II Vertical Electrophoresis Cell Bio-Rad Hercules CA, 150 volts 100 mA 1h) After electrophoresis the gel was soaked in 50% methanol (HPLC grade Fisher Scientific) for 1 h Then the gel was stained with silver (Wray et al 1981) for 15 min in a solution containing 20% silver nitrate 0 36% sodium hydroxide and 14 8 M ammonium hydroxide After staining, the gel was washed with double distilled water (2 x 10 min) The gel was soaked in a developing solution of 1% of citric acid and 38% formaldehyde until bands appear (usually between 10 to 15 min) To stop development, the gel was washed with water and placed in a solution of 50% methanol 10% glacial acetic acid (Fisher Scientific) and 40% double distilled water To determine molecular mass of FSH subunits bands broad-range molecular mass protein standards (7 7 to 204 kDa, Bio-Rad Kaleidoscope prestained molecular weight standards) were loaded onto a different gel and subjected to 15% SDS-PAGE but not stained with silver

Statistical Analyses

Data were analyzed by least squares analysis of variance using the General Linear Models Procedure of the Statistical Analysis System (SAS 1988). Data are presented as least squares means ± SEM of triplicate measurements. Basal secretion and secretion of E_2 and P_4 are expressed as ng/10^5 live cells or as ng/well at the end of the culture period. Basal secretion is defined as the secretion of steroids giving by the priming dose (0.5 ng of FSH/ml) or zero dose of FSH during the different culture periods. To analyze basal secretions of E_2 and P_4, the mathematical model included type of FSH (no FSH, CY-FSH, B-FSH, USDA-FSH), time in culture, and the interaction type of FSH by time. The mathematical model used to analyze the secretion of E_2 and P_4 after FSH treatment and the number of cells at the end of the culture included type of FSH, time in culture (48, 72 and 96 h), dose of FSH, type of FSH by time, type of FSH by dose, time by dose, type of FSH by time by dose interactions as sources of variation. Orthogonal contrasts were used to evaluate the response among the different sources of FSH. Also sequential contrasts (Littell et al., 1991) were used to evaluate the response among doses (0 vs other doses, 0.5 vs 1, 1 vs 2, 2 vs 4, 4 vs 6, 6 vs 8, 8 vs 10, and 10 vs 50) for the different FSH sources tested.

Results

Time Course of Basal Hormone Secretion

Estradiol. There was a significant effect of time ($P < 0.001$) on basal E_2 secretion

(Figure 5-2A). Estradiol decreased from 24 h (14.0 ± 0.2 ng/well) to 48 h (5.0 ± 0.1 ng/well) to 72 h (0.8 ± 0.1 ng/well) and to 96 h (0.3 ± 0.3 ng/well) for all types of FSH. After 24 h of culture, basal secretion of E_2 was the same ($P > 0.8$) for cells that received no FSH (14.7 ± 0.6 ng/well) and cells primed with 0.5 ng/ml CY-FSH (14.1 ± .2 ng/well), whereas cells primed with B-FSH or USDA-FSH tended ($P < 0.1$) to secrete less E_2 than CY-FSH and no FSH-primed cells, but E_2 did not differ ($P > 0.4$) between the B-FSH- (13.8 ± 0.2 ng/well) and USDA-FSH-primed cells (13.4 ± 0.2 ng/well; Figure 5-2A). After 48 h of culture, basal E_2 secretion decreased for all FSH types, but basal secretion was still higher for no FSH and CY-FSH (5.8 ± 0.4 ng/well and 6.9 ± 0.2 ng/well, respectively) than USDA-FSH and B-FSH (4.3 ± 0.2 ng/well and 3.3 ± 0.2 ng/well, respectively; Figure 5.2A). At 72 h of culture, basal E_2 secretion continued to decrease, but was higher for cells that received no FSH or CY-FSH (1.6 ± 0.4 ng/well and 1.2 ± 0.2 ng/well, respectively; Figure 5-2A) than for cells that received USDA FSH or B-FSH (0.7 ± 0.2 ng/well and 0.4 ± 0.2 ng/well, respectively; Figure 5-2A). The same trend was present at 96 h (0.5 ± 0.6 ng/well, 0.4 ± 0.5 ng/well, 0.2 ± 0.7 ng/well and 0.3 ± 0.6 ng/well for no FSH, CY-FSH, USDA-FSH and B-FSH, respectively; Figure 5-2A), but the differences were not significant statistically.

When basal secretion of E_2 was expressed as ng of $E_2/10^5$ live cells, time effect also was significant ($P < 0.001$) with E_2 secretion decreasing from 48 h to 72 h to 96 h for all FSHs (9.4 ± 0.4 ng/10^5 live cells > 2.2 ± 0.4 ng/10^5 live cells > 0.8 ± 0.4 ng/10^5 live cells, respectively). CY-FSH stimulated the highest basal secretion of E_2 at 48 h (17.6 ± 0.7 ng/10^5 live cells; $P < 0.001$; Figure 5-2B), followed by no

FSH primed cells (9 1 ± 0 8 ng/10 ^{5} live cells) basal production of B-FSH (6 9 ± 0 9 ng/10 ^{5} live cells) or USDA FSH primed cells (3 9 ± 1 0 ng/10 ^{5} live cells) were lower and differed ($P < 0.001$) from each other

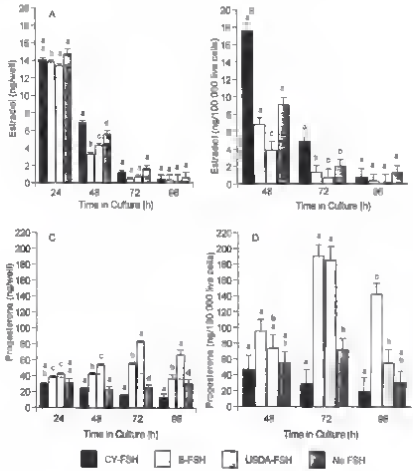

Figure 5-2 Basal secretion of estradiol-17β and progesterone expressed as ng/well (A and C respectively) or as ng/10^5 live cells (B and D respectively) by bovine antral granulosa cells primed with 0 5 ng/ml of CY FSH B-FSH USDA FSH or no FSH during 96 h of culture period Different letters within the same time indicate differences at $P < 0.001$

Estradiol basal secretion per 10^5 live cells at 72 h showed the same pattern with highest production by CY-FSH (4.9 ± 0.7 ng/10^5 live cells $P < 0.01$) and lower E_2 production for no FSH-primed cells (1.3 ± 0.8 ng/10^5 live cells), B-FSH (1.3 ± 0.8 ng/10^5 live cells) and USDA-FSH (0.7 ± 1.0 ng/10^5 live cells, Figure 5-2B). During the last 24 h of culture at 96 h E_2 secretion was low for all FSH-primed cells (1.3 ± 0.8 ng/10^5 live cells, 0.7 ± 1.0 ng/10^5 live cells, 0.3 ± 0.8 ng/10^5 live cells and 0.3 ± 1.0 ng/10^5 live cells for no FSH, CY-FSH, B-FSH and USDA-FSH, respectively, Figure 5-2B) and did not differ between treatments.

Progesterone. Basal P_4 secretion by bovine antral GC was greater than basal E_2 secretion during the 96 h of culture. There was a significant effect of time ($P < 0.001$) on basal P_4 secretion. An FSH by time interaction ($P < 0.001$) was detected for P_4 basal secretion per well or per 10^5 cells (Figure 5-2D). Basal secretion of P_4 declined with time in culture for CY-FSH-primed cells. In contrast, basal P_4 secretion increased with time in culture for cells primed with B-FSH or USDA-FSH (Figure 5-2C). Basal secretion of P_4 by B-FSH- and USDA-FSH-primed cells increased from 24 h (37.7 ± 1.7 ng/well and 41.8 ± 1.9 ng/well respectively; Figure 5-2C) to 48 h (41.9 ± 1.5 ng/well and 53.5 ± 1.8 ng/well respectively, Figure 5-2C) to 72 h (55.2 ± 1.6 ng/well and 82.5 ± 1.9 ng/well respectively, Figure 5-2C). During the last 24 h of culture, basal P_4 secretion induced by B-FSH or USDA-FSH decreased somewhat (36.6 ± 5.1 ng/well and 66.6 ± 6.2 ng/well respectively, Figure 5-2C). Basal P_4 secretion by cells that did not receive FSH did not differ ($P > 0.1$) among different times (24 h: 31.5 ± 5.0 ng/well; 48 h: 23.1 ± 3.6 ng/well; 72 h: 25.2 ± 3.6 ng/well; and 96 h: 31.0 ± 5.1 ng/well; Figure 5-2C).

Basal P_4 secretion expressed as ng of $P_4/10^5$ live cells followed the same pattern At 48 h the lowest basal secretion was induced by CY-FSH (47 0 ± 17 3 ng/10^5 live cells $P < 0$ 004) followed by cells that received no FSH (55 1 ± 14 2 ng/10^5 live cells $P < 0$ 1) USDA-FSH (73 3 ± 17 3 ng/10^5 live cells) and B-FSH (85 6 ± 14 2 ng/10^5 live cells) At 72 h of culture cells that received CY FSH had the lowest basal secretion of P_4 (28 5 ± 17 3 ng/10^5 live cells $P < 0$ 01 Figure 5-2D) followed by cells that received no FSH (71 9 ± 14 2 ng/10^5 live cells) B-FSH (190 1 ± 14 2 ng/10^5 live cells) and USDA-FSH (184 8 ± 17 3 ng/10^5 live cells) had the greatest basal secretion By 96 h basal secretion of P_4 had decreased for all types of FSH (no FSH 30 7 ± 14 2 ng/10^5 live cells CY-FSH 19 4 ± 17 3 ng/10^5 live cells B-FSH 141 7 ± 14 2 ng/10^5 live cells and USDA-FSH 84 8 ± 17 3 ng/10^5 live cells Figure 5-2D) However cells primed with CY-FSH had the lowest secretion ($P < 0$ 01)

Effect of FSH-stimulated Secretion

Estradiol Overall E_2 secretion by antral bovine granulosa cells decreased from 48 h to 72 h to 96 h for each FSH treatment ($P < 0$ 01 Figure 5 3A) Cells treated with CY-FSH had a decrease in E_2 secretion from 18.2 ± 0 3 ng/10^5 live cells at 48 h to 8 4 ± 0 3 ng/10^5 live cells and 3 1 ± 0 4 ng/10^5 live cells at 72 and 96 h respectively (Figure 5-3A) Cells treated with B-FSH and USDA-FSH secreted less E_2 than cells treated with CY-FSH at each time in culture Estradiol secretion (ng/10^5 live cells) at 48 h did not differ among doses of FSH for B-FSH and USDA-FSH

(Figure 5 3B) However cells treated with CY FSH secreted more E_2 ($P < 0.001$) and 1 to 2 ng/ml of CY-FSH stimulated E_2 secretion (Figure 5-3B)

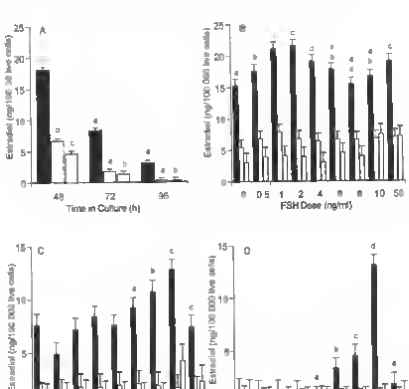

Figure 5-3 Secretion of estradiol-17ß (ng/10^3 live cells) by bovine antral granulosal cells over a 96 h of culture after treatment with different doses of CY-FSH, S FSH and USDA FSH. Main effect of time was significant at $P < 0.001$ (A). Estradiol-17ß secretion during 96 h of culture (A) and in response to doses of FSH at 48 (B), 72 (C) and 96 h (D) respectively. Different letters within the same FSH source indicate differences of $P < 0.001$ (Figures B, C and D)

At 72 h E_2 secretion was increased by 8 and 10 ng/ml of CY-FSH (10.7 ± 1.1 ng/10^3 live cells and 12.8 ±1.0 ng/10^3 live cells $P < 0.01$ Figure 5-3C) whereas at

50 ng/ml of CY-FSH E_2 secretion decreased to basal levels (7 4 ± 1 1 ng/10^5 live cells $P < 0.09$ Figure 5-3C) At 72 h and 96 h of culture B-FSH and USDA-FSH did not stimulate bovine antral granulosa cells to secrete E_2 (Figure 5 3 C and D) By 96 h 6 ng/ml of CY-FSH stimulated E_2 secretion (3 4 ± 1 0 ng/10^5 live cells $P < 0.01$ Figure 5-3D) by bovine antral GC reaching a maximum secretion at 10 ng/ml of CY-FSH (13 1 ± 1 0 ng/10^5 live cells) However a dose of 50 ng/ml of CY-FSH inhibited E_2 secretion (1 8 ± 1 1 ng/10^5 live cells $P < 0.01$)

Overall E_2 secretion expressed as ng/well (Figure 5-4A) also was affected by time ($P < 0.001$) with secretion being higher at 48 h (5 8 ± 0 05 ng/well) than at 72 h (1 1 ± 0 05 ng/well) or 96 h (0 3 ± 0 05 ng/well) Cells treated with CY-FSH secreted more E_2 at 48 h, 72 and 96 h (8 2 ± 0 1 ng/well 1 7 ± 0 1 ng/well and 0 6 ± 0 1 ng/well respectively $P < 0.001$ Figure 5-4A) than B-FSH- (4 4 ± 0 1 ng/well 0 8 ± 0 1 ng/well and 0 3 ± 0 1 ng/well at 48 h 72 h and 96 h respectively Figure 5-4A) or USDA-FSH-treated cells (4 5 ± 0 1 ng/well 0 9 ± 0 1 ng/well and 0.2 ± 0 1 ng/well at 48 h 72 h and 96 h respectively Figure 5-4A) At 48 h there were differences ($P < 0.001$ Figure 5-4B) in E_2 secretion by granulosa cells stimulated by 8 10 and 50 ng/ml of CY-FSH In the case of B-FSH, E_2 secretion increased from 3 1 ± 0 3 ng/well at a dose of 0 ng/ml of FSH to 5 1 ± 0 3 ng/well induced by a dose of 8 ng/ml of B-FSH ($P < 0.06$ Figure 5-4B) USDA-FSH stimulated the secretion of E_2 at doses of 10 ng/ml (9 1 ± 0 3 ng/well) and 50 ng/ml (8 1 ± 0 3 ng/well $P < 0.001$ Figure 5-4B) At 72 h there was an increase in E_2 secretion when GC were stimulated by 8 ng/ml of CY-FSH (2 1 ± 0 2 ng/well Figure 5-4C) this increase was sustained when doses of 10 or 50 ng/ml of FSH were added to

the culture (2.6 ± 0.2 ng/well and 2.5 ± 0.2 ng/well respectively Figure 5-4C). No differences in E_2 secretory response were obtained when different doses of B-FSH were added to the culture (Figure 5-4C) whereas 10 ng/ml of USDA-FSH stimulated an increase of E_2 secretion by bovine antral GC (1.6 ± 0.3 ng/well $P < 0.01$ Figure 5-4C).

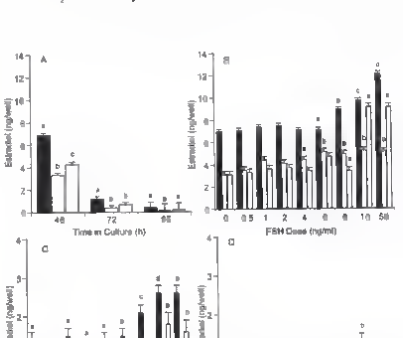

Figure 5-4 Secretion of estradiol (ng/well) by bovine antral granulosa cells over a 96 h of culture in response to different doses of CY-FSH, B-FSH and USDA FSH. Main effect of time was significant at $P < 0.001$ (A). Estradiol-17β secretion during 96 h of culture (A) and in response to doses of FSH at 48 (B), 72 (C) and 96 h (D) respectively. Different letters within the same FSH source indicate differences at $P < 0.001$ (Figures B, C and D).

At 96 h E_2 production by granulosa cells was stimulated by CY-FSH ($P <$

0 001) at doses of 4 (0 4 ± 0 1 ng/well) 6 (0 6 ± 0 1 ng/well) 8 (0 8 ± 1 ng/well) 10 (1 3 ± 0 1 ng/well) and 50 ng/ml (0 6 ± 1 ng/well. Figure 5-4D) Maximal response was detected at 10 ng/ml with a decreased response at 50 ng/ml There were no differences among doses for B-FSH and USDA-FSH at 96 h of culture (Figure 5-4D)

Progesterone There were no significant differences in total P_4 secretion (ng/10^5 live cells) among different FSH sources (Figure 5-5A) at 48 or 96 h However at 72 h B-FSH induced the highest P_4 secretion ($P < 0.001$) At 48 72 or 96 h no differences among doses for different FSH sources were detected (Figure 5 5B C and D) When secretion of P_4 was expressed as ng/well an interaction of FSH by dose by time interaction was detected ($P < 0.001$) There were differences among FSH sources ($P < 0.001$ Figure 5-6A) across times ($P < 0.001$) when P_4 secretion (ng/well) after FSH treatment was examined There was a decrease ($P < 0.001$) in P_4 secretion induced by CY-FSH from 48 h (34 5 ± 1 6 ng/well) to 72 h (23 1 ± 1 6 ng/well) with no changes at 96 h (23 0 ± 1 6 ng/well Figure 5-6A) For the other two FSH sources P_4 secretion increased ($P < 0.001$) from 48 h (B-FSH 53 6 ± 2.2 ng/well and USDA-FSH 59 4 ± 2 6 ng/well) to 72 h (B-FSH 112 6 ± 2,2 ng/well and USDA-FSH 148 0 ± 2 6 ng/well), and decreased ($P < 0.001$) at 96 h (B-FSH 72 6 ± 2.2 ng/well and 114 3 ± 2 6 ng/well Figure 5-6A)

At 48 h P_4 secretion (ng/well) was the same for all doses of CY-FSH (Figure 5-6B) Although P_4 secretion was greater for B-FSH no B-FSH dose effect was detected whereas doses of 10 and 50 ng/ml of USDA FSH induced a response by

by GC (68 7 ± 7 9 ng/well and 100 6 ± 7 9 ng/well respectively $P < 0.002$ Figure 5-8B)

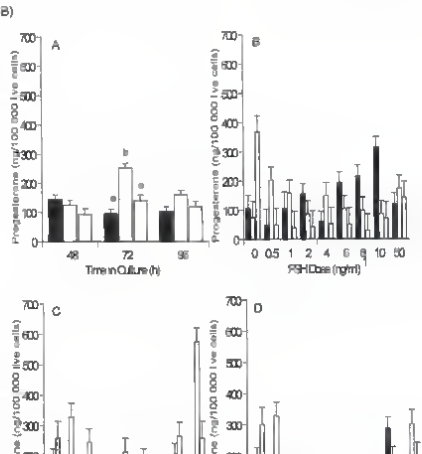

Figure 5-5 Secretion of progesterone (ng/10^5 live cells) by bovine antral granulosa cells over 96 h of culture in response to different doses of CY-FSH, B-FSH and USDA-FSH Progesterone secretion during 96 h of culture (A) and in response to doses of FSH at 48 (ff) 72 (C) and 96 h (D) respectively

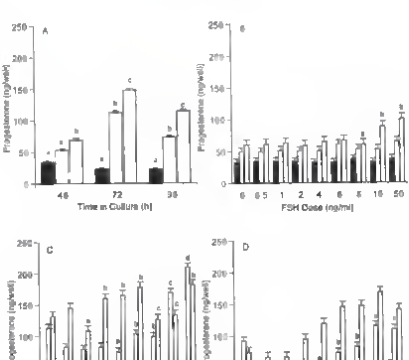

Figure 5-6 Secretion of progesterone (ng/well) by bovine antral granulosa cells over 96 h of culture in response to different doses of CY FSH, B-FSH and USDA FSH. Main effect of time was significant at $P < 0.001$ (A). Secretion of progesterone during 96 h of culture (A) and in response to doses of FSH at 48 (B), 72 (C) and 96 h (D), respectively. Different letters within the same FSH source indicate differences at $P < .001$ (Figures B, C, and D).

At 72 h there were no differences in P_4 secretion among doses of CY FSH except for an increase at 50 ng/ml which stimulated cells to secrete 40.6 ± 5.6 ng/well ($P < 0.001$, Figure 5-6C). B-FSH-stimulated cells secreted more P_4 with secretion further stimulated between 6 to 50 ng/ml and highest secretion of P_4 was

stimulated at 50 ng/ml of B-FSH (209 5 ± 6 5 ng/well P < 0 01 Figure 5-6C)

Secretion of P_4 at 72 h by cells treated with USDA-FSH was high but no consistent dose effect was detected At 96 h P_4 secretion continued to be lowest with CY FSH and a slight stimulatory dose effect in P_4 secretion was detected between doses of 6 to 50 ng/ml of CY FSH (Figure 5-6D) Belgium-FSH-treated granulosa cells secreted more P_4 and doses of 6 8 10 and 50 ng/ml of B-FSH stimulated P_4 secretion Progesterone secretion was greater in granulosa cells treated with USDA-FSH and P_4 secretion increased at doses 6 to 50 ng/ml when compared with all other doses (P < 0 05 Figure 5-6D)

Effect of FSH on Cell Number

Effects of FSH by time interaction were detected on cell number (P < 0 001 Figure 5 7)

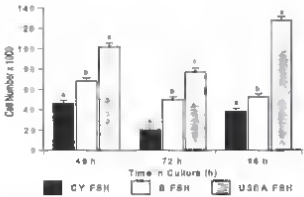

Figure 5 7 Number of bovine antral granulosa cells at the end of 48 72 and 96 h of culture with CY-FSH B-FSH and USDA FSH Different letters indicate differences at P < 0 001 within time

Basically GC number remained constant with CY-FSH treatment However B-FSH and USDA-FSH treatments increased number of cells maintained in culture Clearly number of cells maintained in culture was less than the number seeded (3 x 10^5 live cells) with these losses detected at 48 h This initial loss was attributed to cell loss with change of media at 24 h due to cells not being attached After this period number of cells was maintained within each FSH treatment group and actual number of cells appeared to increase in the USDA-FSH treatment group by 96 h This apparent proliferation did not appear to occur with the other FSHs

Effect of FSH on Cell Morphology

After 24 h of culture in presence of 0 5 ng/ml of CY-FSH granulosa cells maintained their original round shape and started to form cell aggregates forming clumps (light aggregates of spherical granulosa cells) Shape of granulosa cells varied according to the type of steroid secreted (Figure 5-8) Estrogenic granulosa cells treated with CY-FSH at 96 h (Figure 5-8A), were round in shape whereas non estrogenic granulosa cells treated with USDA-FSH at 96 h (Figure 5-8B) had an enlarged fibroblast-like appearance Clumps of granulosa cells were more frequently observed in wells that received CY-FSH and produced more E_2 than those that received B-FSH or USDA-FSH Flattened fibroblast like cells with processes (Figure 5-8B) were more abundant in wells treated with B-FSH or USDA-FSH and this was most evident in cells treated with high doses of FSH (≥ 10 ng/ml)

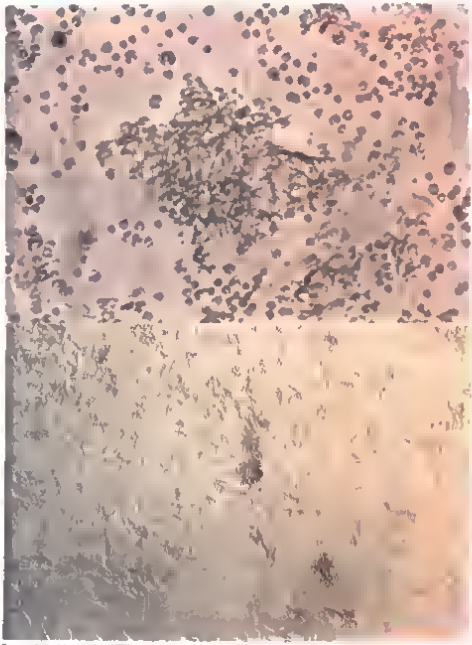

Figure 5.4. Morphological appearance of antral bovine granulosa cells after 24 h of treatment with 4 ng/ml of CY FSH (top panel x400) and 10 ng/ml of USDA FSH (bottom panel x100). Cells were primed with 0.5 ng/ml of the respective FSH for 48 h (top panel) and 72 h (bottom panel).

Gel Electrophoresis

Figure 5-9 shows the results obtained following SDS-electrophoresis of porcine (CY-FSH and B-FSH) and ovine FSH (USDA-FSH). The gel pattern for CY FSH revealed a doublet band at the 600 ng concentration (lane 2) the upper band being darker than the bottom one. On lane 1 there is a very light band, and on lane 3 (700 ng) the band is more intense than lane 1. For B-FSH (lane 4) there are two light bands at the same molecular weight level than for CY FSH (23 kDa). For USDA-FSH (lane 5) the pattern is different. There is a major band at 75 kDa molecular weight which corresponds to BSA present in the preparation and two more bands at higher (> 75 kDa) molecular weights, then at the same level than the other lanes (around 23 kDa molecular weight) there are two light bands that correspond to FSH.

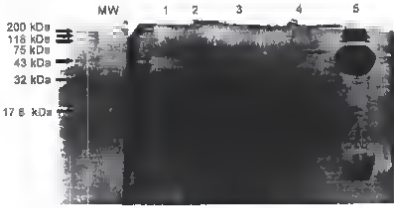

Figure 5-9 One dimensional SDS-PAGE of porcine (CY-FSH and B-FSH) and ovine FSH (USDA-FSH). Lane 1 to 3 correspond to CY FSH (600, 650 and 700 ng/gel lane respectively), lane 4 corresponds to B FSH (600 ng/gel lane) and lane 5 corresponds to USDA-FSH (500 ng/gel lane).

Discussion

Luteinization of cultured bovine granulosa cells treated with high doses of FSH is a common feature (Berndtson et al 1995, Kuren et al 1998). In culture systems that use serum as a supplement, bovine granulosa cells also luteinize spontaneously and lose their ability to produce E_2 in response to FSH (Sirard et al. 1991). It has been shown that E_2 secretion by granulosa cells is lost after the first day of culture (Skinner and Osteen 1988). The present study demonstrated that bovine antral GCs from estrogen active follicles (E_2= 351 ng/ml) can be stimulated by low doses of CY-FSH to produce E_2 in a long-term culture. These results agree with other recent results (Saumande 1991, Berndtson et al. 1995, Rouillier et al 1996, Gutiérrez et al 1997).

The present study demonstrated that the estrogenic response of bovine antral granulosa cells depended upon the source of FSH used. A quadratic dose response was obtained with CY-FSH at 96 h (Figure 5.3D), whereas the other two FSH sources were unable to stimulate an aromatase/estradiol response (Figure 5.3D). A 6 ng/ml dose of CY-FSH was able to stimulate E_2 secretion by antral granulosa cells with maximum secretion occurring at 10 ng/ml of FSH, and a dose of 50 ng/ml inhibited E_2 secretion. Saumande (1991), Berndtson et al. (1995) and Rouillier et al. (1996) reported that the maximal stimulatory dose was between 1 and 2 ng/ml, and doses equal or higher to 10 ng/ml of CY-FSH were inhibitory.

Both Saumande (1991), Rouillier et al. (1996) and the present experiment

utilized the same source of FSH (CY FSH). Probably the difference in dose-response among the studies was due to the type of follicles used in the experiments. In the present experiment, antral granulosa cells were harvested from follicles of cows injected with 25 mg of FSH-P during 3 days after aspiration of a persistent dominant follicle. In experiments of Saumande (1991) and Rouillier et al. (1996), granulosa cells were harvested from follicles of PMSG treated calves. The difference in response to lower or higher doses of FSH could be attributed to differences in half life between PMSG (up to 6 days; Heap and Flint, 1984) and FSH (about 30 min; Heap and Flint, 1984) and that PMSG may have had a carryover residual effect on the cells.

The decrease in E_2 secretion from 48 h to 72 h to 96 h (end of the culture period) is in agreement with previous reports (Berndtson et al., 1995; Rouillier et al., 1996) and is due likely to a reduction in activity of aromatase enzyme that has been observed previously in bovine granulosa cells (Skinner and Osteen, 1988; Rouillier et al., 1996). The shift in the response of antral granulosa cells to different doses of FSH through 96 h of culture period could be due to a change in sensitivity of cells to higher doses of CY-FSH. At 48 h, increased secretion of E_2 by bovine antral granulosa cells occurred at low doses of CY-FSH (e.g., 0.5, 1, and 2 ng/ml). After 72 h of culture, cells were not able to respond to low doses of CY-FSH and required greater doses of FSH (e.g., 6, 8 and 10 ng/ml) to produce E_2. However, the highest dose of 50 ng/ml of CY-FSH was inhibitory, perhaps due to downregulation of the FSH receptor. It is possible that the change in sensitivity is due to a decrease in the

turnover of FSH receptors at the cell membrane level due to internalization of receptors which increases the requirements of FSH to elicit a response

Different types of FSH (ovine FSH Langhout et al., 1991, Spicer and Alpizar 1994 Berndtson et al 1995 Kuran et al 1995 Spicer and Stewart 1996 Kuran et al., 1996 bovine FSH Gutiérrez et al 1997 porcine FSH Saumande 1991 Rouillier et al 1996) have been used to stimulate bovine granulosa cells in culture In the present experiment we used ovine FSH (USDA-FSH) and porcine FSH (B-and CY FSH) Only CY-FSH stimulated GC to produce E_2 during long term culture Several explanations could account for this difference It is possible that there are other proteins present in the preparation For example USDA FSH is provided in a carrier of BSA (10 µg of USDA-FSH is lyophylized with 10 mg of BSA) perhaps BSA interfered with the biological activity of FSH in culture Alternatively BSA may stimulate luteinization and proliferative responses due to potential presence of growth factors and fatty acids On the other hand both CY FSH and B-FSH are apparently very pure preparations Perhaps some isomeric forms of FSH present in CY-FSH are different from those in B FSH like other glycoprotein hormones exists as a family of isohormones which differ in their oligosachande structures including the degree of terminal sialylation and/or sulfation (Ulloa-Aguirre et al., 1995) Structural differences in these isoforms alter their metabolic clearance rates as well as biological and immunological potencies (Ulloa Aguirre et al. 1995) It has been shown that the receptor-binding activity/immunoactivity ratio of the intrapituitary isoforms decreases as the pI of the corresponding isoform declines Studies in the female hamster have demonstrated a close relationship between the

relative sialic acid content of the isoforms and their ability to bind to the receptor (Ulloa-Aguirre et al 1984) Perhaps these structural differences can account for differences in ability of the different FSH sources used in this experiment to induce E_2 secretion by bovine antral granulosa cells Furthermore different preparations of FSH may be more labile to the lyophylization process

It has been shown previously that P_4 secretion by granulosa cells increases with time in culture (Skinner and Osteen 1988) This study showed that basal (Figure 5-2 C and D) and total FSH-stimulated secretion (Figure 5-5A) of P_4 decreased with time when CY-FSH was used to stimulate the cells whereas P_4 secretion per well increased for B- and USDA-FSH The response of bovine antral granulosa cells to different doses of CY-FSH in terms of P_4 secretion was not as clear as the response in terms of E_2 secretion at the end of the 96 h culture period (Figures 5-3 and 5-5D) Cells treated with CY-FSH had a quadratic response to dose of FSH showing a maximal E_2 response at 10 ng/ml with a decrease in E_2 secretion at a dose of 50 ng of FSH/ml No dose effect of CY-FSH B-FSH or USDA-FSH was detected on P_4 secretion In contrast B-FSH and USDA-FSH failed to stimulate E_2 secretion Our study suggests that the steroidogenic nature of the cells or the degree of differentiated characteristics of luteinization differed between FSH types used

The number of cells at the end of the 96 h culture period was lower for CY-FSH than for B-FSH and USDA-FSH treatments Apparently, the latter two FSH preparations induced the granulosa cells to differentiate into a cell type with a more fibroblastic appearance that secretes P_4 Cells treated with USDA-FSH formed

monolayers and assumed a fibroblastic appearance with cell processes whereas cells treated with CY FSH were tightly packed with a round shape and grew in multilayered aggregates as reported previously in the rat (Knecht et al 1981) and cow (Gutiérrez et al 1997) The dynamics of cellular proliferation in our culture were difficult to quantify since appreciable cell loss occured early (e g by 48 h) for all groups and cell numbers at each time were less than cells plated Round cells typical of CY-FSH would be more prone to be lost with medium change compared to granulosa cells with fibroblastic-like appearance Increased cell number at 96 h for the USDA FSH may have represented a proliferative phase Thus with the experimental approach utilized we were unable to demonstrate a stimulatory effect of FSH on proliferation of bovine granulosa cells in culture Similar results were reported by Langhout et al (1991) Gong et al (1993) and Rouillier et al (1995) On the other hand Gutiérrez et al (1997) reported that FSH in the presence of 10 ng/ml of insulin stimulated proliferation of cells from small medium and large follicles as measured by cell number at the end of the culture Similar results have been reported by Campbell et al (1998) with ovine granulosa cells proliferation *in vitro* Skinner and Osteen (1988) reported that granulosa cells isolated from large follicles had a slightly lower plating efficiency and greater loss in cell number between days 3 and 6 of culture This could be the case in our study where the source of granulosa cells were large follicles (≥ 9 mm)

Implications

The present experimental approach demonstrated a differential response of

bovine antral granulosa cells to different sources of FSH and that there is a shift in the estrogenic response of bovine antral granulosa cells to doses of CY-FSH with time in culture. Granulosa cells from bovine ovarian follicles did not proliferate in culture in response to CY-FSH and shape of granulosa cells in culture varied according to the type of steroid secreted. Estrogenic granulosa cells from follicles ≥ 9 mm exposed to CY-FSH had a round shape and grew in multilayered aggregates or clumps, whereas nonestrogenic granulosa cells had an enlarged fibroblastic-like appearance. Based on these results we can conclude that this bovine antral granulosa cell culture system can be used to study the regulatory factors controlling secretion of E_2. For example effects of dimeric and monomeric forms of inhibin that are present during transitory phases of follicular dominance can be evaluated as to their ability to regulate E_2 secretion in granulosa cells. Based upon the results in the present chapter it is recommended that E_2 secretion be evaluated as ng of E_2 per 10^5 live cells since cell loss appeared to differ between types of FSH that influenced morphogenic appearance in culture. Furthermore dose effects of CY-FSH on E_2 secretion appeared to be more apparent when expressed as ng of E_2 per 10^5 live cells.

CHAPTER 6
EFFECT OF BOVINE FOLLICULAR FLUID FROM DAY 5 DOMINANT AND DAY 12 ATRETIC DOMINANT FOLLICLES ON IN VITRO ESTRADIOL-17β SECRETION BY BOVINE ANTRAL GRANULOSA CELLS

Introduction

Ovarian folliculogenesis is a dynamic process characterized by a marked proliferation and differentiation of the somatic cell components of the follicle and is associated with development of a group of follicles at various stages of maturation from which a species-specific number of follicles are selected for continued growth (Armstrong and Webb 1997). The developing follicle provides the optimal environment for the maturation of the oocyte in readiness for its subsequent ovulation and fertilization. Gonadotropins are the primary regulators of folliculogenesis. They regulate ovarian folliculogenesis via classic endocrine mechanisms. In addition, intraovarian mechanisms are involved in the control of folliculogenesis (Ireland 1987; Tonetta and di Zerega 1989). The concept of intraovarian control of ovarian function arose in the 1970s (Armstrong and Webb 1997). These ovarian factors regulate both secretion of FSH and follicular responsiveness to gonadotropins (Findlay 1993).

Ovarian follicles produce an array of locally acting peptides and growth factors that interact directly with the same cell type from which they are produced

or with other cell types within the developing follicle to stimulate or attenuate the cellular response to gonadotropins. The processes of recruitment, selection and dominance are controlled by the interaction of endocrine signals and locally produced ovarian growth factors that are associated with morphological and functional changes in granulosa and theca cells (Armstrong and Webb, 1997).

Inhibins, activin, insulin-like growth factor I (IGF-I) and their binding proteins have direct and indirect effects on granulosa and theca cells that can modulate follicular development and steroidogenesis. Inhibin is a heterodimeric glycoprotein composed of an α subunit and a β subunit which are not linked covalently. The β subunit occurs in two different forms, termed βA and βB, giving rise to inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$) on combination with the α subunit (Findlay et al., 1993; Rose and Gomes-Das, 1996). Inhibins have autocrine, paracrine and endocrine effects. Inhibin is a glycoprotein produced primarily in granulosa cells (Henderson and Franchimont, 1983), and preferentially inhibits pituitary FSH synthesis and/or secretion (De Jong, 1988; Glencross et al., 1984) by inhibiting expression of the gene encoding the β subunit of FSH (Beard et al., 1989). Inhibins through their systemic actions have an important role in determining species-specific ovulation rates (e.g., ovulation of only one follicle in cattle; Taya et al., 1996; Scanlon et al., 1993).

Nine different forms of biologically and immunologically active inhibin (ranging from 29 to 160 kDa) have been isolated from bovine follicular fluid of dominant follicles (Good et al., 1995). The predominant inhibin forms are free α subunits and large (> 34 kDa) inhibin forms, rather than the fully processed inhibin

form (Ireland et al 1994) Inhibin increases *in vitro* LH induced androgen synthesis of bovine theca cells (Wrathall and Knight 1995) and immunoactive inhibin secretion is stimulated by androstened one testosterone and E_2 (Wrathall and Knight, 1993) However FSH had no effect on basal immuncective inhibin secretion by bovine (Wrathall and Knight, 1993) and ovine (Campbell et al 1996) granulosa cells Activin inhibited both LH stimulated and E_2-stimulated androstenedione secretion by bovine thecs cells (Wrathall and Knight, 1995) Furthermore, activin reversed the positive effect of inhibin on basal LH-stimulated and E_2-stimulated androstenedione secretion but did not affect basal steroid output (Wrathall and Knight 1995)

Simultaneous addition of follistatin and activin reversed the inhibitory effect of activin but did not modify the effects of inhibin These observations era indicative of opposing intrafollicular paracrine roles for granulosa cell-denved inhibin and activin in modulating thecal cell responses to gonadotropins and steroids in the bovine ovary Epidermal growth factor and transforming growth factor-α stimulated marked increases in proliferation of granulose cells from both small and large ovine follicles and a concomitant inhibition of E_2 and inhibin synthesis (Campbell et al 1994)

Probably a local feedback loop exists within individual follicles involving a sequential change of inhibins activins and their binding proteins which determines the different fates of selected and unselected follicles that develop in the same systemic environment of gonadotropins and growth factors (Roche 1996) Intrafollicular contents of inhibin (34 kDa) activin and IGF binding protein 2 (IGFBP-

2) are related inversely to E_2-producing capacity of dominant and subordinate follicles (Roche 1996). It has been shown (Yuan et al. 1998) that dominant follicles expressed abundant IGF-I and IGF-II mRNA but failed to express IGFBP 2 which is a binding protein associated with atresia (Monget and Monniaux 1995). On the other hand, subordinate follicles expressed lesser amounts of IGF I and IGF-II but abundant IGFBP 2. Thus, activin, inhibin and growth factors have key local negative roles on gonadotropic action that may control processes of follicle selection and dominance (Roche 1996).

The precise physiological role of inhibin in folliculogenesis is unknown. Results of several studies support both endocrine (Scanlon et al. 1993; Glencross et al. 1994) and local (Knight et al. 1989; Guilbault et al. 1993; Ireland and Ireland 1994; Ireland et al. 1994) roles of inhibin in regulation of dominant follicles during the bovine estrous cycle. Sato et al. (1982) suggested that an inhibin-like substance present in bovine follicular fluid inhibits FSH action at the ovary through binding to the FSH receptor on the granulosa cell. More recently, a report by Schneyer et al. (1991) indicated that inhibin α subunits bind to FSH receptor sites and inhibit FSH bioactivity in rat granulosa cells. To understand the local role of inhibin, an experiment was designed to evaluate the effect of inhibins present in bovine follicular fluid of day 5 dominant and day 12 atretic dominant follicles on *in vitro* E_2 secretion by bovine antral granulosa cells.

Materials and Methods

Inhibin Immunoaffinity Column

An inhibin immunoaffinity column was developed to obtain bovine follicular fluid (bFF) free of inhibins and to isolate the monomeric and multimeric forms of inhibin in follicular fluid (Good et al 1995) A partially affinity purified rabbit antiserum against bovine α_c^{1-26} gly-tyr (Good et al 1995) provided by Dr J J Ireland (Michigan State University East Lansing, Michigan) was cross- linked to a HighTrap™ Protein G Sepharose® High Performance Column (Pharmacia Biotech Inc Piscataway NJ) The protein G column was equilibrated with three bed volumes of washing/binding buffer (10 mM sodium phosphate pH 7 0 150 mM sodium chloride 10 mM ethylenediaminetetraacetic acid [EDTA]) Prior to the coupling, antiserum was centrifuged (Microcentrifuge Model 235B Fisher) for 1 min at 13,000 x *g* One ml of anti-inhibin antiserum was applied to the column and incubated for 1 h at 4 C The column was washed with 10 ml of washing/binding buffer to remove unbound antibody and then washed with 10 ml of 0 2 M triethanolamine (TEA) pH 8 5 To cross-link the antibody the column was incubated for 30 min at room temperature (RT) with 10 ml of a solution containing TEA (0 2 M pH 8 5) plus freshly made dimethylpimelimidate (10 mg/ml pH 8 5 adjusted with NaOH) This allows optimal spatial orientation of antibodies and maximum antigen binding efficiency (Schneider et al 1982) The reaction was stopped by washing the column with10 ml of 0.2 ethanolamine (ETA), pH 8 0 followed by incubation for 2 h at room temperature At the end of the incubation

period the column was washed with 10 ml of washing/binding buffer and the column kept at 4°C until use

Pools of Bovine Follicular Fluid

Pools of bovine follicular fluid (bFF) were prepared from day 5 dominant follicles (DF) and day 12 atretic DFs (ADF) Cows (n=25) were synchronized with an injection of GnRH (Buserelin 9 µg i m Receptal® Hoechst-Roussel Agri Vet Sommerville NJ) followed 7 days later with an injection of prostaglandin (PG) $F_{2\alpha}$ (25 mg i m Lutalyse Pharmacia & Upjohn Co Kalamazoo MI) At estrus cows were assigned either to the day 5 DF (n=13 6.4 ml of FF) or day 12 ADF (n=10 9 7 ml of FF) groups

On day 5 and 12 for the respective groups first wave dominant follicles were aspirated via transvaginal ultrasound guided aspiration utilizing an Aloka Echo-Camera SSD-500 unit (Aloka Co. LTD Japan) equipped with a 5 0 MHz transvaginal convex array transducer and needle guide Epidural anesthesia was induced with 5 ml of 2% lidocaine and the perineal region was scrubbed and disinfected The lubricated transducer with needle guide was inserted deep into the vagina A 17-gauge 60 cm single channel sterile needle fitted with a 19-gauge 2 5 cm disposable tip was used in the needle guide to puncture the vaginal wall and peritoneum By manipulation of the reproductive tract per rectum ovaries were positioned and held firmly in front of the transducer in close apposition to the vaginal wall After aligning the on-screen needle travel indicator line with the follicle the needle was advanced through the wall of the vagina into ovarian stroma and

through the wall of the follicle. As soon as the tip of the needle was seen to enter the follicle, follicular contents were aspirated into a sterile plastic tube using a suction unit (Pioneer Medical Inc., Madison, CT.) preset to a flow rate of 20 ml/minute at a preset pressure of 100 mm Hg. After collection, follicular fluid was centrifuged (2000 x *g*, 15 min, 4°C) to remove cellular debris and kept at 4°C throughout processing until frozen at -20°C.

Dextran-coated charcoal was prepared by mixing 25 mg of dextran (65-600 molecular weight, Sigma, St. Louis, MO) and 250 mg of activated charcoal (100-400 mesh, Sigma, St. Louis, MO) in 100 ml of double distilled water for 16 h. After mixing, the solution was centrifuged at 2 619 x *g* for 30 min, supernatant discarded and pellets dried overnight in an oven at 35 °C. Prior to the use of follicular fluid endogenous steroids were removed from pools of bFF by overnight treatment with dextran-coated charcoal (5 mg/ml). At the end of overnight incubation, follicular fluids were centrifuged thrice (12 365 x *g* for 30 min). After charcoal treatment, both pools of bFF (day 5= 5.2 ml and day 12= 6 ml) were dialyzed (molecular weight cutoff: 3500; day 5= 5.2 ml and day 12= 7.8 ml) against Tris buffered saline (TBS: 0.1 M Tris [hydroxymethyl] Aminomethane, 1.5 M sodium chloride), pH 7.6 for 24 h, changing TBS every 6 h.

To verify steroids concentrations E_2 and progesterone [P_4] were extracted from steroid-stripped free follicular fluid as described by Thatcher et al. (1994b; E_2) and Knickerbocker et al. (1986; P_4) and assayed using validated radioimmunoassays (Thatcher et al., 1994b). To determine if use of dextran-coated charcoal to remove steroids from bFF would not remove inhibins, follicular fluid was

collected from ovaries retrieved from the slaughter house Ovaries were collected and brought to the laboratory in about 2 h They were transported at room temperature in an insulated container with sterile saline solution [0 9% NaCl] containing 1% penicillin-streptomycin [Sigma St Louis MO]) Upon arrival ovaries were washed with medium for ovary collection at 37°C (Chapter 5) FF collected by aspiration and treated as follows dextran-coated charcoal (5 mg/ml) overnight and dialyzed for 24 h non charcoal-treated and dialyzed for 72 h or 144 h dialyzed for 144 h and charcoal-treated for 5 h or charcoal treated overnight Samples of the different treated follicular fluids (50 µg/lane) were subjected to a nonreducing one-dimensional 10% SDS PAGE (see page 8)

Experimental follicular fluids aspirated from day 5 DF and day 12 ADF were pooled because the amount of follicular fluid from each individual follicle was not sufficient to accommodate the preparation of inhibin free bFF and immunoaffinity enriched inhibin in sufficient quantity for various treatments in cultures of bovine antral granulosa cell Pools of bFF (day 5 DF and day 12 ADF) were sub-divided into two portions one portion (day 5= 2 5 ml day 12= 3 ml) was diluted 1 2 in TBS buffer and treated in order to have follicular fluid without inhibin forms (FF without inh) and an inhibin enriched fraction (inh-eluate) the other portion (day 5= 2 5 ml day 12= 4 8 ml) was left intact (FF complete [FFC]) The diluted follicular fluid (day 5= 5 ml and day 12= 6 ml) was applied to the inhibin immunoaffinity column in aliquots of 1 ml and eluted as follows. 1ml of bFF diluted 1 2 was applied to the column and incubated at 4 °C for 2 h After incubation the column was eluted with three 1 ml volumes of ice cold TBS buffer pH 7 6 Each 1 ml of the eluate fraction

was collected separately and represented bFF without inhibins. The inhibins bound to the column then were eluted with four 1 ml volumes of 50 mM sodium acetate pH 2.5, and each 1 ml collected separately. Before collection, 100 µl of 5 M NaOH were added to bullet tubes to neutralize the eluates containing 50 mM sodium acetate, pH 2.5. These fractions represent the eluate of immunoabsorbed inhibins (inh-eluate). After collection of inh-eluate, the column was washed with 2 ml of double distilled water followed by 2 ml of TBS buffer. This procedure was repeated to prepare the entire day 5 DF (5 ml at a dilution of 1:2) and day 12 ADF FF (8 ml at a dilution of 1:2).

Eluate fractions of inhibin (day 5= 10 ml, day 12= 12 ml) were dialyzed (molecular weight cutoff 3500) for 48 h against TBS buffer, pH 7.6, with changes in dialysis buffer every 6 h. After dialysis, protein concentration was measured in all fractions (FFC, FF without inh fractions, and inh-eluate fractions) using the bicinchinonic acid (BCA, Smith et al., 1985) assay with BSA as a standard. Based on protein concentrations, first and second ml fraction of FF without inh eluate, and second and third fraction ml of inh-eluate were pooled.

Electrophoresis and Western Blotting Procedures

Samples of day 5 DF and day 12 ADF fractions designated as bFFC, FF without inhibins and inh-eluate (50 µg protein/gel lane) were mixed in 4X (12.5 µl) sample loading buffer (8% of sodium dodecyl sulphate [SDS, BDH Laboratory Supplies, Poole England], 4% glycerol, 250 mM Tris-HCl pH 6.8, and bromophenol

blue solution until solution becomes medium dark blue color [0 002%] and double distilled water to a final loading volume of 50 µl) without β-mercaploethanol (non reducing conditions) For FF without inh the loading of the sample was based on amount of protein equal to FFC (e.g., 50 µg protein) and by volume of fraction alone equivalent to the volume of FFC necessary to have 50 µg of protein In the case of day 5 FF without inh the volume was equal to 5 5 µl of FF diluted 1 10 The volume of day 12 FF without inh was equal to 5 8 µl of FF diluted 1 10 Samples were boiled for 5 min and subjected to one-dimensional 10% SDS-PAGE (Laemmli, 1970) using a Bio-Rad mini gel apparatus (Mini-Protean II Vertical Electrophoresis Cell, Bio-Rad, Hercules, CA, 150 volts, 100 mA, 1 h electrophoresis buffer 192 mM glycine, 25 mM Tris, 0 1% SDS) To determine molecular mass of inhibin bands broad-range molecular mass protein standards (7 7 to 204 kDa Bio-Rad Kaleidoscope prestained molecular weight standards) were loaded (10 µl) onto a single lane per gel and subjected to 10% SDS-PAGE concomitant with the bFF fractions but were not mixed in sample loading buffer and not boiled

After electrophoresis, gel and membranes were incubated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8 1-8 4) for 30 min Proteins on the gel were transferred using a Trans-blot® semi-dry transfer cell (Bio-Rad, 20 volts, 400 mA, 45 min) to a Hybond™ ECL™ nitrocellulose membrane (Amersham Life Science, Little Chalfont, Buckinghamshire, England) Each membrane was blocked with 2% gelatin (Fisher Scientific, Fair Lawn, NJ) in TBS-Tween 20 (TBST, 0 1% Tween 20 plus TBS) for 2 h at RT and washed twice (1 x 15 min, 1 x 5 min)

in TBST The membrane was incubated for 2 h at RT with an inhibin antiserum (diluted 1 50 in 5% non-fat milk [Blotting Grade Blocker Non-Fat Dry Milk Bio Rad Hercules CA] The antiserum was generated in rabbit against bovine α_{1-26} gly-tyr (Good et al 1995) and was absorbed with plasma from a 3-week old female calf Briefly 100 µl of the inhibin antiserum were diluted in 1 ml of plasma from a 3-week old female calf incubated for 2 h at 4°C in a rotating device centrifuged at 13 000 x g (Microcentrifuge Modal 235B Fisher) for 5 min and the supernatant retained as absorbed anti inhibin antiserum (diluted 1 10)

After incubation with the anti-bovine inhibin-α_{1-26} gly-tyr (Good et al 1995) membranes were washed thrice in TBST (1 x 15 min 2 x 5 min) incubated at RT for 1 h with a second antibody (peroxidase labeled goat anti-rabbit IgG antibody NIF 284 Amersham Life Science) diluted 1 12 000 in TBST containing 2% gelatin + 0 1% of goat normal serum Following incubation the membrane was washed in TBST (1 x 15 min 4 x 5 min each) and protein bands identified by chemiluminescence The membrane then was incubated for 1 min with equal 4 ml volumes of detection reagent 1 and detection reagent 2 (ECL™ Western Blotting Analysis System Amersham International plc Little Chalfont Buckinghamshire England) Detection reagents were drained from the membrane and the membrane wrapped in plastic saran wrap before placement into a single cassette (Kodak X-Omatic Eastman Kodak Rochester NY) for 1 min with autoradiography film (Reflection™ NEN® Research Products Autoradiography film NEN™ Life

Science Products Boston MA) Developing of autoradiography film was performed in a Konica X-ray film processor QX 70 (Konica Corporation Japan)

Granulosa Cell Culture System

Antral bovine granulosa cells (GC) were cultured in a defined medium using the system previously described (Chapter 5) Briefly cycling cows (n= 6) were synchronized with an injection of GnRH (Buserelin 8 µg I m Receptal® Hoechst-Roussel Agri Vet Sommerville NJ) followed 7 days later with an injection of $PGF_{2\alpha}$ (25 mg I m Lutalyse Pharmacia & Upjohn Kalamazoo MI) On day 6 of the synchronized cycle one Synchromate B (SMB Sanofi Animal Health Inc Overland Park KS) implant was inserted and two injections of $PGF_{2\alpha}$ were given (25 mg at 7 00 h 15 mg at 19 00 h) On day 10 the DF was aspirated (as described earlier) and two days later on day 12 treatment with FSH-P (FSH-P™ Schering-Plough Animal Health Kenilworth NJ) began and the SMB implant was replaced with a new SMB implant Cows (n= 5) received 28 mg of FSH given in decreasing doses every 12 h (A M /P M = 6/6 5/5 and 3/3 mg per injection) On day 15 the SMB implant was removed and ovaries were collected at slaughter (Figure 5-1 Chapter 5)

Antral GC were harvested from all follicles ≥ 9 mm and cultured (Chapter 5) in serum-free medium in 48-well plates (Costar Cambridge MA) for 48 h at 37°C gassed with 5% CO_2 and 95% air Wells were precoated with 3 µg/cm^2 of fibronectin (for 30 min at 37°C) before seeding of the cells ($3x10^5$ cells/well) Cells

were cultured in serum-free medium Ham's F-12 buffered with $NaHCO_3$ (1 176 mg/ml) and Hepes (20 mM) pH 7 4 supplemented with insulin (50 ng/ml) androstenedione (10^{-7} M) human transferrin (partially iron-loaded 10 µg/ml) ascorbic acid (17 6 µg/ml) gentamicin (20 µg/ml) and nystatin (4 µg/ml) To optimize the *in vitro* secretion of E_2 medium was supplemented with a priming dose of 0 5 ng/ml of FSH (porcine FSH [CY-FSH]= 41 x NIH-FSH-P1 LH activity < 0 5% gift from Dr C Y Combarnous INRA Nouzilly France) for the first 24 h of culture During the second 24 h cells were treated with 0 5 or 10 ng/ml of CY-FSH and each dose of the different fractions of bFF as shown on Table 6-1 (n = 2 wells per each combination of FSH dose and fraction dose) Dose 1 of day 5 DF inh-eluate was equivalent to 15 625 times the amount of α- and dimeric forms of inhibin (2.14 µg) in 10 µl of bovine follicular fluid from estrogen-active follicles (α-inhibins 214 1 µg/ml [0 28% of total protein] and dimeric inhibins 0 866 µg/ml [0 0013% of total proteins] Guilbault et al 1993) Dose 2 was equivalent to 244 1 times the amount of α- and dimeric forms of inhibin in 10 µl of bovine follicular fluid from estrogen-active follicles (Guilbault et al 1993) Dose 1 of day 12 ADF eluate inh was equivalent to 15 625 times the amount of α- and dimeric forms of inhibin in 10 µl of bovine follicular fluid from estrogen-inactive follicles (α-inhibins 90 5 µg/ml [0 10% of total protein] dimeric inhibins 2 96 µg/ml [0 0034% of total proteins] Guilbault et al 1993) Dose 2 was equivalent to 244 1 times the amount of α- and dimeric forms of inhibin in 10 µl of bovine follicular fluid from estrogen inactive follicles (Guilbault et al 1993)

Table 6-1 Doses of different fractions of bovine follicular fluid of day 5 dominant and day 12 atretic dominant follicles

	Day 5 Dominant Follicle		Day 12 Atretic Dominant Follicle	
Fractions	Dose 1	Dose 2	Dose 1	Dose 2
Follicular Fluid Complete	10 µl (763 8 µg)	20 µl (1527 6 µg)	10 µl (864 2 µg)	20 µl (1728 4 µg)
Follicular Fluid without Inhibins	94 4 µl (763 8 µg)	188.8 µl (1527 6 µg)	96 µl (864 2 µg)	192 µl (1728 4 µg)
Inhibins-eluate	10 15 µl (33 55 µg)	158 55 µl (524 21 µg)	3 44 µl (13 6 µg)	53 7 µl (216 12 µg)

In order to test that E_2 is synthesized from androstenedione (A_4) by bovine antral granulosa cells in culture wells without A_4 but with FSH (0 5 and 10 ng/ml FSH n=4 per dose) also were included A series of control incubations were conducted to examine the effects of no follicular fluid (e g culture media alone and effects of different amounts of TBS buffer used as a diluent for the follicular fluid fractions (e g FFC FF-inh inh-eluate)) The volumes of TBS tested on E_2 secretion by granulosa cells are shown on Table 6-2

Table 6-2 Effects of TBS volume added to culture medium (500 µl total volume) on E_2 secretion by bovine antral granulosa cells in culture

Medium	Volume of TBS used in FF fractions as diluent
500 µl	0 µl
446 3 µl	53 7 µl (volume of dose 2 inh-eluate of day 12)
405.6 µl	94.4 µl (volume of dose 1 FF without inh of day 5)
404 0 µl	96 µl (volume of dose 1 FF without inh of day 12)
341 4 µl	158.6 µl (volume of dose 2 inh-eluate of day 8)
311 2 µl	188 8 µl (volume of dose 2 FF without inh of day 8)
308 0 µl	192 µl (volume of dose 2 FF without inh of day 12)

Statistical Analyses

Data were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS 1988). Data are presented as means ± SEM of duplicate measurements. Secretion of E_2 and P_4 are expressed as ng/10^5 live cells at the end of the culture period. For purposes of data presentation, E_2 and P_4 concentrations will be expressed as ng/10^5 live cells (Chapter 5). The mathematical models used to analyze the data are presented in Table 6-3.

Because the four way interactions were significant, each day (day 5 DF and day 12 ADF) was analyzed separately to further understand the higher order interactions. The reduced mathematical models are described in Table 6-3.

Table 6-3. Mathematical models used to analyze the secretion of estradiol and progesterone by antral granulosa cells at the end of the 48 h culture period.

Full Model		Reduced Model	
Sources	d f	Sources	d f
Dose of CY-FSH	1	Dose of CY-FSH	1
Day of DF	1	Fraction	2
Fraction of follicular fluid	2	Dose of fraction	1
Dose of fraction	1	Dose of CY-FSH x fraction	2
Dose of CY-FSH x day of DF	1	Dose of CY-FSH x dose of fraction	1
Dose of CY-FSH x fraction	2	Fraction x dose of fraction	2
Dose of CY-FSH x dose of fraction	1	Dose of CY-FSH x fraction x dose of fraction	2
Day of DF x fraction	2		
Day of DF x dose of fraction	1		
Fraction x dose of fraction	2		

Table 6-3 continued

Full Model		Reduced Model	
Sources	d.f.	Sources	d.f.
Dose of CY-FSH x day of DF x fraction	2		
Dose of CY-FSH x day of DF x dose of fraction	1		
Day of DF x fraction x dose of fraction	2		
Dose of CY-FSH x fraction x dose of fraction	2		
Dose of CY-FSH x day of DF x fraction x dose of fraction	2		
Error	24	Error	12

Orthogonal contrasts were used to evaluate responses. The following contrasts were tested: dose of CY-FSH (0.5 ng/ml vs 10 ng/ml); fraction of FF (FFC vs FF without inhibins + inh-eluate and FF without inhibins vs inh-eluate) and dose of fraction (dose 1 vs dose 2). Contrasts were also examined for the interactions that were significant.

These analyses do not include cow in the model regarding the FF preparation or cow as a source of variability relative to source of granulosa cells. As described earlier, FF from individual cows at day 5 or at day 12 were pooled to provide sufficient follicular fluid for processing of fractional components for testing in culture. Thus, significant differences regarding day of DF and FF fractions represent differences between the pools of follicular fluid from day 5 dominant and day 12 atretic dominant follicles. A total of 13 cows contributed to the day 5 FF pool and 10 cows contributed to the day 12 FF pool. Statistical differences are interpreted to represent a true physiological difference between the follicular fluid

pools (day 5 vs day 12) which represents the average of all cows within the pool At risk is the possibility that a few cows within a respective pool may be outliers that account for the difference in response between the follicle pools Hopefully such an effect is minimized by the large number of animals within a pool at a specific stage of dominant follicle development

Granulosa cells of follicles $\geq$ 9 mm were pooled across five cows that underwent the follicular superinduction scheme in order to provide sufficient number of granulosa cells for the biological test system It is not possible to utilize as many experimental treatments and replicates on granulosa cells prepared from each donor cow separately Furthermore it is extremely costly Differences among cows were minimized since follicles designated for aspiration had to be $\geq$ 9 mm and healthy in appearance (well vascularized and translucent)

Results

Validation of Dextran Coated Charcoal Treatment

An immunoblot of bFF from ovaries collected at the slaughterhouse and subjected to charcoal treatment and dialysis is shown in Figure 6-1 There were no major differences among the different lanes for the molecular forms of bovine inhibin after the respective treatments Dimeric and monomeric forms of immunoabsorbed reactive bovine inhibin were evident on the blots (> 160 110 77 58 49 kDa) and agrees with those reported by Good et al (1995) and da la Sota (1995) Apparently the 34 kDa form was not present in the FF because lane 1 (FF without any treatment Figure 6 1) does not show any band at that molecular weight

size When FF was dialyzed for 0 72 or 144 h without dextran-coated charcoal treatment E_2 decreased from 2 8 µg/ml to 85 8 ng/ml and 0 8 ng/ml respectively Because E_2 concentration was considered high even after 144 h of dialysis charcoal treatment for 16 h plus dialysis for 24 h was the approach chosen (lane 2) Concentrations of E_2 and P_4 in dextran-charcoal treated bFF after charcoal treatment for 16 h and dialyzed for 24 h were < 0 39 ng/ml and < 0 31 ng/ml respectively Thus dextran-coated charcoal effectively removed E_2 and P_4 from follicular fluid

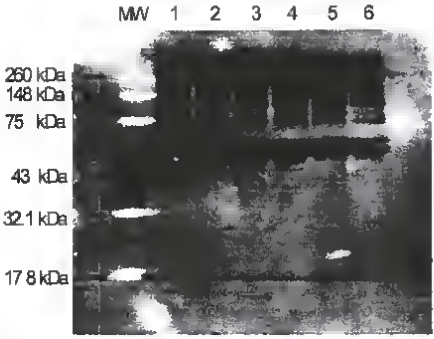

Figure 6-1 Immunoblot analysis of bFF from slaughterhouse ovaries Fifty µg of protein from each sample were subjected to a non-reducing one-dimensional 10% SDS-PAGE and immunoblot analysis using a partially affinity purified rabbit antiserum against bovine inhibin-α_{1-26} gly-tyr (Good et al 1995) Lane 1= bFF without any treatment lane 2= bFF charcoal-treated for 16 h and dialyzed for 24 h lane 3= bFF dialyzed for 72 h lane 4= bFF dialyzed for 144 h lane 5= bFF dialyzed for 144 h and charcoal-treated for 5 h and lane 6= bFF dialyzed for 144 h and charcoal-treated for 16h

Concentrations of total proteins were very high (Table 6-4) in follicular fluid and did not differ appreciably between untreated and treated FF that was subjected to dextran-coated charcoal stripping and dialysis. There were no obvious changes in immunoblotting intensity among the six treatment preparations as well, indicating no preferential removal of immunoinhibin active bands among treatments.

Figure 6-2 (Commassie blue staining of the gel) shows no differences in the array and intensity of proteins among the different treatments both at high and low molecular weights. For example, the 29 kDa protein was evident among all treatments. At these high total protein concentrations in FF, the amount of preferential removal of protein at various sizes appear to be minimal (Figure 6-2). Furthermore, the immunoreactive proteins appear to be uniform. Thus, pre-preparations of FF for subsequent fractionation did not appear to cause a total loss of protein or differential loss of selected protein.

Table 6-4. Protein concentration (mg/ml) in dextran-coated charcoal treated follicular fluid from slaughter house ovaries.

Treatment	Protein concentration (mg/ml)
Follicular fluid without any treatment	69.4 ± 1.00
Follicular fluid charcoal-treated for 18 h + dialysis for 24 h	66.3 ± 0.75
Follicular fluid no charcoal-treated + dialysis for 72 h	61.8 ± 0.87
Follicular fluid no charcoal-treated + dialysis for 144 h	66.5 ± 0.96
Follicular fluid dialyzed for 144 h + charcoal-treated for 5 h	65.2 ± 0.70
Follicular fluid dialyzed for 144 h + charcoal-treated for 18 h	63.5 ± 0.80

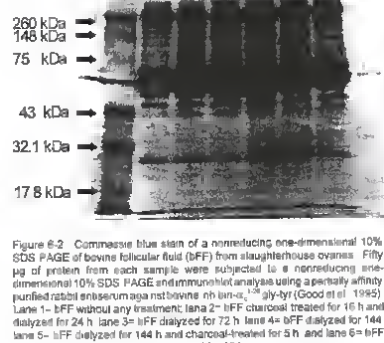

Figure 6-2 Coomassie blue stain of a nonreducing one-dimensional 10% SDS PAGE of bovine follicular fluid (bFF) from slaughterhouse ovaries Fifty µg of protein from each sample were subjected to a nonreducing one-dimensional 10% SDS PAGE and immunoblot analysis using a partially affinity purified rabbit antiserum against bovine inh α_{1-26} gly-tyr (Good et al 1995) Lane 1= bFF without any treatment; lane 2= bFF charcoal treated for 16 h and dialyzed for 24 h lane 3= bFF dialyzed for 72 h lane 4= bFF dialyzed for 144 lane 5= bFF dialyzed for 144 h and charcoal-treated for 5 h and lane 6= bFF dialyzed for 144 h and charcoal treated for 16 h

Protein concentrations of experimental pools of day 5 dominant and day 12 atretic dominant follicles FF before charcoal treatment were 83 2 mg/ml and 83 6 mg/ml for day 5 and day 12 respectively After charcoal treatment protein concentration did not change (day 5 81 3 mg/ml and day 12 80 7 mg/ml) Following dialysis protein concentrations were 76 382 mg/ml and 86 429 mg/ml for day 5 DF FFC and day 12 ADF FFC respectively This reflects some loss of protein ≤ 3 500 kDa Figure 6-3 shows the elution profile for protein content of FF without inh and inh-eluate of day 5 DF (Figure 6-3A) and day 12 ADF (Figure 6-3B)

Fractions 1 and 2 of FF without inh and fractions 2 and 3 of inh-eluates were pooled to make up the FF without inh and inh-eluate material for testing in culture.

For both day 5 and day 12 follicular pools there was not a 100% recovery of protein from the column (Table 6-5). For day 5 DF 43.25% of the initial protein was recovered in the fractions whereas 66.93% of protein was recovered for the column runs of day 12 FF pool. This loss in protein concentration is due to several reasons. There was always a loss of some fluid when the follicular fluid was loaded into the column. Because of the viscous nature of FF some foaming occurred and when air was expelled from the syringe to avoid adding air to the column several microliters were lost. Also when FF was loaded into the column, the last drops of buffer associated with displacement of the bed volume of the column contained some of the FF protein being loaded. Protein concentration was measured in some of these fractions and contained between 2.9 to 7.5 mg/ml of protein. These various fractions contributed to an incomplete recovery of total proteins.

Western Blot Analysis of Inhibin in Follicular Fluid

Immunoblot analysis of the experimental fractions using an bovine inhibin-α_c[1-26] gly-tyr antibody (Good et al. 1995) indicated that some inhibin forms (49-110 kDa forms) were removed when immunoreactive bands of day 5 and 12 FF without inh were compared to their respective follicular fluids that were complete (bFFC). In the inh-eluate fractions there is an enrichment of the 34 and 49 kDa proteins (Figure 6-4).

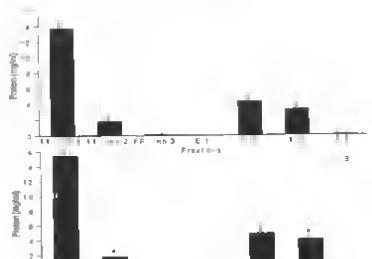

Figure 6-3 Elution profiles for protein concentration of FF without inhibins and inhibins-eluate fractions from day 5 DF and day 12 ADF. Asterisk indicates the fractions with the highest protein concentration that were chosen to conform the pool from the respective day

Table 6.5 Protein concentration (mg/ml) and protein recovery (mg) from follicular fluid of day 5 dominant and day 12 atretic dominant follicles (1 ml FF diluted 1:2) when processed (1 ml) through the immunoaffinity column

	Day 5 dominant follicle		Day 12 atretic dominant follicle	
Fraction	PR (mg)	% Recov	PR (mg)	% Recov
FF added	42.8		41.2	
FF-Inh 1	10.8 ± 0.130	25.25	15.4 ± 3.60	37.45
FF-Inh 2	1.35 ± 0.100	3.15	1.8 ± 0.03	4.44
FF-Inh 3	0.12 ± 0.020	0.28	0.47 ± 0.03	1.14
Inh-eluate 1	0.02 ± 0.001	0.05	0.07 ± 0.03	0.17
Inh-eluate 2	3.33 ± 0.430	7.77	4.86 ± 0.70	11.85
Inh-eluate 3	2.63 ± 0.100	5.51	4.06 ± 0.19	9.86
Inh-eluate 4	0.06 ± 0.010	0.14	0.79 ± 0.22	1.92
Total	18.51	43.25	27.47	66.83

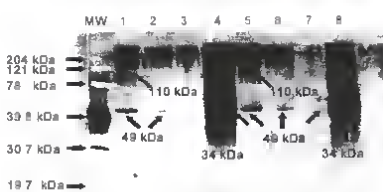

Figure 6-4 Different molecular masses (kDa) of immunoactive inhibin bFF (50 µg of protein) for day 5 DF FF after charcoal treatment and dialysis before passage through the immunoaffinity column (lane 1) FF without inhibins (loaded by equal amount of protein as FF before passage through the column lane 2 or loaded by equal volume to volume of FF that contains 50 µg of protein lane 3) Inhibins-eluate day 5 DF FF (50 µg of protein lane 4) Day 12 ADF FF after charcoal treatment and dialyzed before passage through the inhibin immunoaffinity column (lane 5) FF without inhibins (loaded by equal amount of protein as FF before passage through the column lane 6 or loaded by equal volume to volume of FF that contains 50 µg of protein lane 7 Inhibins-eluate day 12 ADF FF (50 µg of protein lane 7#)

The predominant forms detected on day 5 DF FFC (lane 1) were 49 110 and > 160 kDa whereas a lesser amount of the 98 kDa form was present When FF without inh was loaded as equal amount of protein to day 5 FFC (lane 2) the bands that correspond to the inhibin forms in FFC were much lighter (49 110 > 160 kDa form) and when the loading of FF without inh was done by equal volume (lane 3) to volume of day 5 FFC only one region (> 160 kDa) was present

On the day 5 inh-eluate (lane 4) band that correspond to the > 160 kDa 49 kDa and 34 kDa forms were evident Day 12 ADF FFC also showed bands at 49 110 and > 160 kDa and a less intense band at 89 kDa When FF without inhibins

(lane 6) was loaded by equal amount of protein to day 12 FFC the same bands that were present in FFC were present but less intense When the loading of the sample was based on volume (same volume as day 12 FFC lane 7) the only proteins present were those > 160 kDa forms In lane 8 (inh-eluate day 12) there were bands at > 160 kDa 48 kDa and a less intense band at 34 kDa In summary 49 88 and 110 kDa forms were markedly removed from day 5 FFC by the immunoaffinity column and the > 160 kDa forms were decreased The 34 kDa 48 kDa and 88 kDa forms were present on the inh-eluate In the case of day 12 FFC the 49 88 110 and > 160 kDa forms were present All of these proteins were markedly reduced in the FF without inh fraction and were enriched in the inh-eluate These forms of inhibin identified in the present experiment coincide with those reported by Good et al (1995) and de la Sota (1995) using a bovine antiserum against bovine inhibin α_c^{1-26} gly tyr and a mink antiserum against bovine inhibin α_c^{1-26} gly-tyr respectively

Figure 6-5 shows the Commassie blue stain of the array of proteins present in FFC FF without inh and FF inh-eluate of day 5 DF (lanes 1 2, 3 and 4) and day 12 ADF(lanes 5, 6, 7 and 8) There are distinct differences among the lanes Lanes 4 and 8 which represent immunoabsorbed proteins show a markedly different array of proteins compared to FFC (lanes 1 and 5) and FF without inh (lanes 2 and 6) The selective removal of only immunoreactive inhibin forms (lanes 4 and 8, Figure 6-4) is consistent with a similar array of commassie blue stained proteins (Figure 6-5 lanes 4 and 8) Although FFC (lanes 1 and 5) and FF without

inh (lanes 2 and 6) have some pattern of proteins the pattern of immunoabsorbed protein is different (Figure 6-4) indicating selective removal of inhibin forms

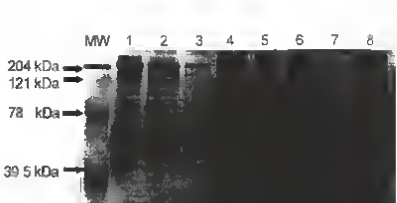

Figure 6-5 Commassie blue stain of a non reducing one-dimensional 10% SDS-PAGE of bFF from day 5 DF and day 12 ADF and their different fractions after passage through the inhibin immunoaffinity column Day 5 DF FF after charcoal treatment and dialysis before passage through the immunoaffinity column (50 µg of protein lane 1) FF without inhib ns (loaded by equal amount of protein as FF before passage through the column lane 2 or loaded by equal volume to volume of FF that conta ns 50 µg of protein lane 3) Inhib na-eluate day 5 DF FF (50 µg of protein lane 74) Day 12 ADF FF after charcoal treatment and dialyzed before passage through the inhibin immunoaffinity column (lane 5) FF without inh bins (loaded by equal amount of prote n as FF before passage through the column lane 6 or loaded by equal volume to volume of FF that contains 50 µg of protein lane 7). Inhibins-eluate day 12 DF FF (50 µg of protein, lane 8)

Effect of Bovine Follicular Fluid on FSH-Stimulated Estradiol-17β Secretion

Estradiol-17β Androstenedione is the precursor for estrogen synthesis When A_4 was not added to the culture concentration of E_2 in medium conditioned by antral granulosa cells was below the sensitivity of the assay (0 39 ng/ml Chapter 5) for either the 0 5 and 10 ng/ml dose of CY-FSH Thus addition of A_4 substrate to

granulosa cells is essential for E_2 secretion since thecal cell components that produce androstenedione are absent from the culture system

A dose of 10 ng/ml of CY FSH produced more E_2 (10^5 live cells) than a dose of 0 5 ng/ml of CY FSH ($P < 0 03$) A volume of TBS effect was not detected ($P > 0 10$ Table 6-5)

Table 6-6 Estradiol (ng/10^5 live cells) secretion by bovine antral granulosa cells in absence of follicular fluid and in presence of different volume of TBS

Volume of TBS	Estradiol (ng/10^5 live cells)
No follicular fluid	19 9 ± 1 9
53.7 µl	13 6 ± 1 9
94 4 µl	14 7 ± 1 9
96 0 µl	16.2 ± 1 9
158 6 µl	15 5 ± 1 9
188.8 µl	18.4 ± 1 9
192 0 µl	23.1 ± 2.3

There was no evidence that increased TBS diluent would adversely decrease E_2 secretion via a dilution of nutrients in the culture medium (Table 6-6) Thus follicular fluid fraction effects (positive or negative) will be due to the protein components in the fraction not the TBS diluent In general E_2 secretion at 0 5 (11 7 ng/100 000 live cells) and 10 ng/ml of CY-FSH (26 3 ng/100 000 live cells) was greater ($P < 0 06$) in the absence of follicular fluid proteins than in the presence of follicular fluid proteins Thus subsequent analyses are comparisons among follicular fluid fractions which contain proteins that appear to be stimulatory or inhibitory to E_2 secretion

Table 6-7 shows the analysis of variance for analysis of data utilizing the full model The four way interaction (dose of CY-FSH x day of follicular fluid x fraction x dose of fraction) for E_2 and P_4 concentrations and E_2 P_4 ratio in ng/10^5 live cells was significant Therefore to examine this in more detail seperate analyses were done for granulosa cell incubations treated with FF from day 5 dominant (Table 6-8) and day 12 (Table 6-9) ertetic dominent follicles

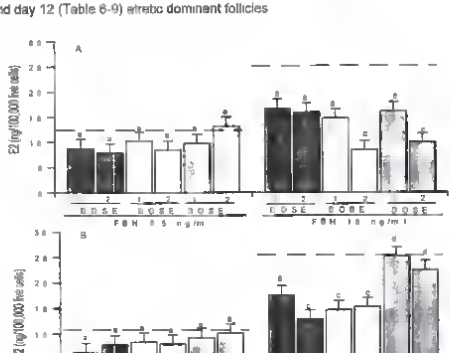

Figure 6-6 Secretion of estradiol (ng/10^3 live cells) by bovine antral granulosa cells over 48 h of culture in response two doses of CY FSH (0 5 and 10 ng/ml) and two doses of different fractions of day 5 FF (panel A) and day 12 ADF follicular fluid (panel B) Different letters within the same day and cate differences at P < 0 0001 Dotted lines represent E_2 secretion in the absence of follicular fluid

A significant three way interaction (dose of CY-FSH by fraction by dose of fraction $P < 0.0001$ Figure 6-6A) was detected for E_2 secretion (ng/10^5 live cells) by antral granulosa cells in the presence of day 5 FF fractions There were no differences among experimental fractions and doses of experimental fractions on E_2 secretion when cells were treated with 0 5 ng/ml of CY-FSH However E_2 secretion was higher ($P < 0.001$) with the 10 ng/ml dose of CY FSH Dose 1 (18 7 ± 0 9 ng/10^5 live cells) and 2 (18 0 ± 0 8 ng/10^5 live cells) of day 5 FFC in presence of 10 ng/ml of CY-FSH had no affect on E_2 secretion Dose 2 of day 5 FF without inh (8 43 ± 0 8 ng/10^5 live cells) decreased E_2 secretion ($P < 0.001$) Thus factors other than inhibin in day 5 FF decrease E_2 secretion by granulosa cells Dose 2 of inhibins-eluate from day 5 DF FF inhibited ($P < 0.001$ Figure 6-6A) E_2 secretion (8 8 ± 0 8 ng/10^5 live cells) in culture Thus inhibin enriched fraction of FF collected from day 5 DF decreased E_2 secretion It is possible that the 49 kDa form that is enriched in this fraction inhibits secretion of E_2 by granulosa cells

In the presence of follicular fluid fractions from day 12 ADF a dose of 10 ng/ml of CY-FSH stimulated a higher secretion of E_2 than a dose of 0 5 ng/ml of CY-FSH ($P < 0.001$ Figure 6-6B) In contrast to day 5 FFC day 12 FFC at dose 2 inhibited E_2 secretion and this degree of inhibition was comparable to the day 12 ADF FF without inh fractions at either dose 1 and dose 2 Thus removal of the inhibin forms increased the negative effect of FF on E_2 secretion Inhibins-eluate at both doses in presence of 10 ng/ml of CY-FSH markedly stimulated granulosa cells to secrete E_2 in culture and no differences were detected in the stimulatory response between the doses of inhibins-eluates (Figure 6-6B) In day 12 FF of atretic follicles there appear to be a form of inhibin that stimulated E_2 secretion

However this stimulation is not evident when mixed with other follicular components that are inhibitory in follicular fluid

Table 6-7 Analysis of variance for E_2 and P_4 concentrations and $E_2:P_4$ ratio (ng/10^5 live cells) in cultured bovine antral granulosa cells treated with follicular fluid from day 5 dominant and day 12 atretic dominant follicles (full model)

Source	df	E_2	P_4	E_2:P_4
Dose of CY-FSH	1	0.0001	0.0001	0.0001
Day of DF	1	0.5403	0.0040	0.1251
Fraction of follicular fluid	2	0.0012	0.0001	0.0163
Dose of fraction	1	0.0008	0.0121	0.0008
Dose of CY-FSH x day of DF	1	0.5730	0.1328	0.9455
Dose of CY-FSH x fraction	2	0.0470	0.0105	0.0283
Dose of CY-FSH x dose of fraction	1	0.0011	0.0503	0.0067
Day of DF x fraction	2	0.0367	0.1370	0.0311
Day of DF x dose of fraction	1	0.3541	0.3177	0.4547
Fraction x dose of fraction	2	0.0025	0.0118	0.0417
Dose of CY-FSH x day of DF x fraction	2	0.0712	0.2414	0.0884
Dose of CY-FSH x day of DF x dose of fraction	1	0.3186	0.5029	0.3104
Day of DF x fraction x dose of fraction	2	0.0189	0.1913	0.0123
Dose of CY-FSH x fraction x dose of fraction	2	0.0047	0.8859	0.0328
Dose of CY-FSH x day of DF x fraction x dose of fraction	2	0.0405	0.0448	0.0260
Error	24			

Table 6-6 Analysis of variance for E_2 and P_4 concentrations and $E_2:P_4$ ratio (ng/10^5 live cells) in cultured bovine antral granulosa cells treated with follicular fluid from day 5 dominant follicle (reduced model)

Source	df	E_2	P_4	$E_2:P_4$
Dose of CY-FSH	1	0.0001	0.0001	0.0001
Fraction	2	0.0148	0.0071	0.0001
Dose of fraction	1	0.0011	0.3880	0.0003
Dose of CY-FSH x fraction	2	0.0002	0.1238	0.0001
Dose of CY-FSH x dose of fraction	1	0.0004	0.4795	0.0007
Fraction x dose of fraction	2	0.0404	0.0635	0.0004
Dose of CY-FSH x fraction x dose of fraction	2	0.0047	0.03494	0.0022
Error	12			

Progesterone There were significant differences ($P < 0.001$ Table 6-6 Figure 6-7A) between doses of CY-FSH in P_4 secretion expressed as ng/10^5 live cells for experimental fractions from day 5 dominant and day 12 atretic dominant follicles The dose of 10 ng/ml of CY-FSH was inhibitory to P_4 secretion by antral granulosa cells exposed to follicular fluid fractions from either stage follicle (day 5 or day 12, $P < 0009$) There were no differences in P_4 secretion between doses of FFC and FF without inh when a dose of 0.5 ng/ml of FSH was added to the culture However dose 2 of inh-eluate stimulated ($P < 0.06$) P_4 secretion (137.2 ± 10.2 ng/10^5 live cells) compared to dose 1 (94.7 ± 10.2 ng/10^5 live cells Figure 6-7A) At the low dose of 0.5 ng/ml of CY FSH inhibin enriched fractions of day 5 DF appeared to stimulate luteinization during the 24 h treatment period (24 to 48 h) in culture There were no differences in P_4 between doses among fractions of

follicular fluid or their doses for day 5 dominant follicles at a dose of 10 ng/ml of CY-FSH

Table 6-9 Analysis of variance for E_2 and P_4 concentrations and $E_2:P_4$ ratio (ng/10^5 live cells) in cultured bovine antral granulosa cells treated with follicular fluid from day 12 atretic dominant follicle (reduced model)

Source	df	E_2	P_4
Dose of CY-FSH	1	0.0001	0.0001
Fraction	2	0.0096	0.0001
Dose of fraction	1	0.5637	0.0001
Dose of CY-FSH x fraction	2	0.1082	0.0012
Dose of CY-FSH x dose of fraction	1	0.2578	0.0011
Fraction x dose of fraction	2	0.8778	0.0418
Dose of CY-FSH x fraction x dose of fraction	2	0.5602	0.0073
Error	12		

When granulosa cells were incubated with CY-FSH at a concentration of 0.5 ng/ml, dose 2 of each FF fraction (FFC, FF without inh, Inh-eluate) stimulated ($P < 0.0001$) P_4 secretion (Figure 6-7B). This was not evident at the higher dose of 10 ng/ml of CY-FSH, except that dose 2 of inh-eluate induced a greater secretion ($P < 0.01$) of P_4 (81.1 ± 3.5 ng/10^5 live cells) than dose 1 (59.2 ± 3.5 ng/10^5 live cells). Thus, the intrafollicular environment of a day 12 ADF appears to stimulate P_4 secretion or luteinization when exposed to a low dose of CY-FSH. This was not evident for follicular fluid fractions collected from a day 5 DF that is more estrogenic.

Estradiol:Progesterone ratio. There was a significant ($P < 0.002$) three way interaction for $E_2:P_4$ ratio (ng/10^5 live cells, Figure 6-8A) for day 5 dominant follicle

FF There were not differences in E_2 P_4 ratio among the different fractions and their doses added to the culture in presence of a dose of 0 5 ng/ml of CY-FSH When a dose of 10 ng/ml of FSH was added E_2 P_4 ratio was higher ($P < 0.001$) for all fractions regardless of fraction dose Dose 2 of FF without inhibins (0 24 ± 0 02) and inh-eluate (0 20 ± 0 02) had lower ($P < 0.004$) E_2-P_4 ratios than their respective one doses (FF without inh 0 40 ± 0 02, inh-eluate 0 40 ± 0 02) This further supports presence of and an enrichment in inhibitory factors to E_2 secretion in these follicular fluid components from day 5 DF

As for the day 5 dominant follicle FF pool there was a higher E_2-P_4 ratio ($P < 0.001$ Figure 6-8B) when 10 ng/ml of CY FSH were added to the granulosa cell culture regardless of the follicular fluid fraction added from day 12 stretic dominant follicle There were not differences in E_2 P_4 ratio between doses among fractions in presence of 0 5 ng/ml of CY-FSH Estradiol P_4 ratio was lower ($P < 0.1$) when dose 2 of FFC (0 3 ± 0 03) and inh-eluate (0 28 ± 0 03) were added to the cells than dose 1 of the same fractions (FFC= 0 38 ± 0 03 Inh-eluate= 0 43 ± 0 03) The E_2 P_4 ratio was decreased in the FF without inh and there were no differences in E_2-P_4 ratio between doses of FF without inhibins

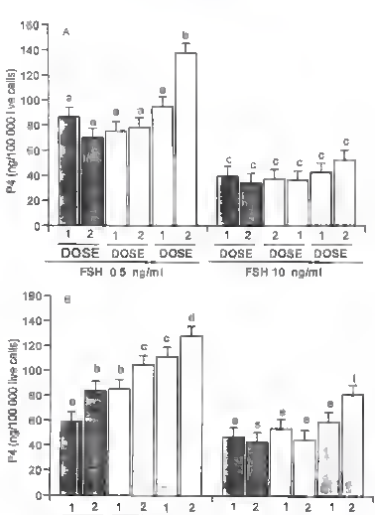

Figure 6.7 Secretion of progesterone (ng/10^5 live cells) by bovine antral granulosa cells over 48 h of culture in response to two doses of CY-FSH (0.5 and 10 ng/ml) and two doses of different fractions at day 5 DF (panel A) and day 12 ADF follicular fluid (panel B). Different letters within the same day indicate differences at $P < 0.01$

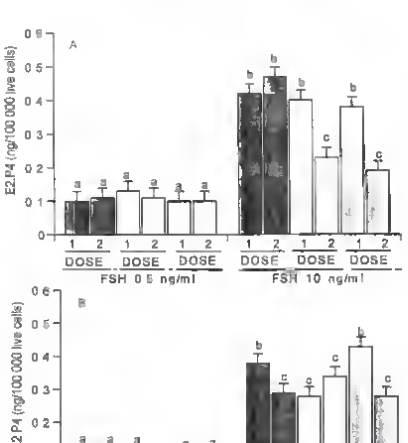

Figure 6-8 Estradiol progesterone ratio (ng/10^3 live cells) in culture medium by bovine antral granulosa cells over 48 h of culture after 24 h of treatment with two doses of CY FSH (0.5 and 10 ng/ml) and two doses of different fractions of day 5 DF (panel A) and day 12 ADF (panel B) follicular fluid. Different letters within the same day indicate differences at $P < 0.01$

Discussion

Changes in amounts and proportions of the different molecular forms of inhibin (de la Sota 1995 Sunderland et al. 1996) and expression of inhibin/activin mRNA for α β_A and β_B subunits (Ireland and Ireland 1994) are related to physiological stages of follicle growth. There are distinct patterns in molecular forms of inhibin (Guilbault et al. 1993 de la Sota 1995 Sunderland et al. 1996) in follicular fluid of dominant (e.g. day 5 DF) or atretic follicles (e.g. day 12 atretic DF). Therefore the role of the inhibin forms may be different according to the stage of dominant follicle development. De la Sota (1995) reported that approximately 25% of inhibin forms present in FF are monomeric forms (α-inhibins) whereas 75% are dimeric forms of inhibin. At day 12 absolute amounts of the 34 kDa form in the atretic dominant follicle increased two fold compared to the day 5 DF.

In the present experiment, amounts of inhibin forms in the pool of FF for day 5 DF and day 12 ADF were not quantified. However the effects of immuno-enriched forms of inhibins in FF on bovine antral granulosa cells in culture were measured between day 5 and day 12 dominant follicles. Day 5 DF appears to have factors that inhibit E_2 secretion which are enriched in FF without inh fraction and in the inh-eluate fraction (Figure 6-6A). Dose 2 of both FF without inh and FF inh-eluate fractions decreased E_2 secretion. These inhibitory components were enriched in the FF without inh and inh-eluate fractions compared to FFC doses. It has been shown that other growth factors (e.g. TGF-α. Roulliér et al. 1997) and other proteins such as IGFBPs (Monget and Monniaux 1995) tissue inhibitor of

matalloproteinase (Roche 1996) and hsp90 (Roche 1996) are present in follicular fluid and have a negative effect on E_2 secretion by antral granulosa cells. In the present study inhibitory effects on E_2 secretion were not observed with a low dose of CY-FSH (0.5 ng/ml) suggesting an interaction with FSH-induced actions. Perhaps a dose of 10 ng/ml of CY-FSH is sufficient to compete with an intra follicular inhibitor for the FSH receptor on granulosa cells. However at the higher doses of FF without inh and FF inh-eluate from day 5 DF, the inhibitors decrease FSH binding and reduce E_2 secretion. This may be the case for an inhibin form in the inh-eluate since inhibin or an α-inhibin form has been suggested to compete for the FSH receptor (Sato et al. 1982; Schneyer et al., 1991). Furthermore hsp90 recently has been shown to be present in FF of bovine (Dinancourt personal communication) and ovine (Dinancourt and Catelli unpublished data) follicles and it may possibly inhibit aromatase activity. Perhaps hsp90 may be a candidate protein that contributes to the inhibitory effect of day 5 and day 12 follicular fluid fractions that contain reduced amount of inhibin (e.g. FF without inh) that inhibits E_2 secretion.

In contrast to day 5 there was a stimulation in E_2 secretion (ng/10^5 live cells) by inh-eluate of day 12 ADF. This differential stimulation was even evident with day 12 ADF follicular fluid since FF without inh was inhibitory to E_2 secretion. Day 12 ADF has a higher proportion of the mature 34 kDa form of inhibin (de la Sota 1995) and it has been hypothesized (Baird and Smith 1993) that increased production of E_2 is in part dependent on the action of inhibin at the intrafollicular level to stimulate LH-induced androgen production (Findlay 1993; Hillier et al.,

1991 Wrathall et al 1995) Therefore a higher proportion of the 34kDa form in the inh-eluate of day 12 than in FF without inh or FFC could explain this difference However present results would suggest an alternative stimulatory mechanism independent of androgen production since the present test system does not include theca cells and androgen substrate is provided Perhaps a direct stimulus in aromatase activity has occurred The lower secretion of E_2 induced by day 12 ADF FF without inh and FFC could be due to other products such as TGF-α hsp90 IGFBPs etc Roullier et al (1997) reported that addition of bFF to granulosa cell culture suppressed FSH-induced E_2 production and that immunoneutralization of TGF-α in bFF restored E_2 secretion to levels obtained in the absence of bFF but these effects are not mediated through the FSH receptor (Legault et al 1997) Concentrations of TGF-α in the pools of FF from day 5 dominant and day 12 atretic dominant follicles used in this experiment were measured by radioimmunoassay (Dr Gregory S Schultz Department of Obstetrics and Gynecology University of Florida) Day 12 FF had a higher concentration (5 41 ng/ml) than day 5 FF (2 53 ng/ml) It is possible that TGF-α present in bFF adversely influenced secretion of E_2 (Roullier et al 1997)

The decrease in E_2 secretion by dose 2 of day 5 DF inh-eluate could be due to higher concentrations of the 49 kDa monomeric form that is present in higher amounts in follicular fluid of day 5 DF compared to day 12 ADF (de la Sota 1995) This difference in concentration as determined by quantification of immunoblots utilizing different antiserum (anti bovine inhibin $\alpha_c^{1\text{-}26}$ gly tyr raised in mink de la Sota 1995) from the present experiment (anti bovine inhibin $\alpha_c^{1\text{-}26}$ gly-tyr raised in

rabbit; Good et al 1995). It has been suggested (Sato et al 1982, Schneyer et al 1991) that an α-inhibin form could bind to FSH binding sites on granulosa cells and interfere with the stimulatory action of FSH.

Bovine antral granulosa cells produced less P_4 in response to the higher 10 ng/ml dose of CY-FSH in the presence of different FF fractions from day 5 DF and day 12 atretic dominant follicles. It is likely that the lower secretion of P_4 was associated with a higher estrogenic capacity of the cells in response to a higher dose of CY-FSH in culture.

Estradiol P_4 ratio is generally regarded as an index of estrogenic capacity of granulosa cells. Higher ratios are associated with higher estrogenic potential of the cells. The increase in E_2 P_4 ratio or enhanced estrogenic activity when exposed to 10 ng/ml of CY-FSH occurred in incubation regardless of whether fractions were from day 5 dominant or day 12 atretic dominant follicles (Figure 6-6A and B). Evidence for autoregulation involving intrafollicular components is supported by the observation that inh-eluate of day 12 ADF stimulated E_2 secretion. However, the effect of these immunoactive inhibins were not expressed when mixed with whole follicular fluid (e.g., FFC) that contained components that were inhibitory to E_2 secretion (e.g., FF without Inh day 12 ADF).

Implications

There are differences among the different fractions of FF from day 5 DF and day 12 ADF to regulate E_2 secretion or aromatase activity in bovine antral granulosa

cells These differences are attributed particularly to the different forms of inhibin and their proportions in follicular fluid of at day 5 and day 12 There is an inhibin-like factor in FF of day 12 ADF that stimulates E_2 secretion This may be the dimeric 34 kDa enriched form of inhibin There is an alternative form of inhibin (49 kDa form perhaps) at day 5 and possible other factors (e g hsp90 EGF/TGF-α) that inhibit E_2 secretion in response to 10 ng/ml of CY-FSH The balance of these factors may control estrogenic activity of the dominant follicle in response to gonadotropins during different functional stages of follicle dominance

CHAPTER 7
GENERAL DISCUSSION AND CONCLUSIONS

A precise understanding of the processes involved in the growth and differentiation of ovulatory follicles in cattle is a prerequisite for development of new techniques and improvement of already existing regimes for estrus synchronization ovulation and superovulation In cattle folliculogenesis can be divided into two parts according to the follicular requirements for gonadotropins a basal folliculogenesis which can proceed in the absence of gonadotropins and a tonic folliculogenesis which requires gonadotropins (Driancourt 1991)

Follicular development in cattle occurs in waves usually involving either two or three waves per estrous cycle (Pierson and Ginther 1984 Sirois and Fortune 1988 Savio et al 1988) Each wave consists of three phases recruitment, selection and dominance During this last phase one follicle grows and through the secretion of endocrine and paracrine factors inhibits the growth of subordinate follicles in the cohort The mechanism by which a dominant follicle is selected and continues to grow while subordinate follicles regress is unknown Also the mechanism(s) by which the dominant follicle regulates growth of the subordinate follicles is not clear

A series of experiments were designed to test *in vivo* the effects of ablation

of the first wave dominant follicle on dynamics of follicular growth and CL development and follicle ablation on follicle development and embryo production in response to a superovulatory treatment. A bovine granulosa cell culture system was developed to test, *in vitro*, the effects of various inhibin forms present in follicular fluid from day 5 dominant and day 12 atretic dominant follicles on estradiol secretion of antral granulosa cells. The dominant follicle of the first wave was chosen because is one of the most predictable events in follicular dynamics during the estrous cycle in cattle (Badinga et al., 1992; Fortune, 1993).

In chapters 3 and 4, the effect of the ablation of the first wave dominant follicle was examined under two different scenarios. In chapter 3, the dominant follicle ovulated after an injection of 3,000 IU of hCG. An accessory CL was formed in all heifers that received hCG, and as a consequence, P_4 levels between days 9 and 17 of the estrous cycle were higher than in the control group. This difference was probably due to a greater growth of the original CL in treated heifers as a consequence of the luteotropic effect of hCG (Schmitt et al., 1996a) and additional P_4 secretion by the accessory CL (Schmitt et al., 1996b). There were no carry-over effects of the hCG injection that influenced CL development during the following estrous cycle.

Human Chorionic Gonadotropin acutely affected follicular dynamics by ovulating the first wave dominant follicle on day 5. All hCG-treated heifers had three follicular waves during the experimental estrous cycle. Recruitment and emergence of the second wave dominant follicle was earlier. It has been shown that a surge of FSH precedes emergence of a follicular wave (Adams et al., 1992) and removal

of the ovary beening the dominant follicle triggers an increase in FSH concentrations (Badinga et al 1992) Removal of the dominant follicle advances emergence of the next follicular wave (Ko et al 1991) In the present experiment, size and lifespan of the second wave dominant follicle were reduced This may be due to higher plasma P_4 concentrations which will induce follicle turnover (Savio et al 1993 Burke et al 1994 Thatcher et al 1994a Kinder et al 1996) due to a reduction in LH secretion (Kinder et al 1996) As a consequence of the shorter lifespan of the second wave dom n ant follicle the third wave dominant follicle emerged earlier and lested longer in the ovary before ovulation Dominant follicles were selected on days 3 or 4 of their respective waves as reported earlier (Ginther et al 1996)

Heifers with three spontaneous follicular waves during the estrous cycle had greater plasma P_4 concentrations and an estrous cycle with a longer duration than heifers with two spontaneous follicular waves Higher concentrations of P_4 could contribute to a higher turnover of the second dominant follicle as a consequence of negative feedback of P_4 on LH secretion that decreases LH pulsatility (Kinder et al 1996) and permits development of a third follicular wave The duration of the luteal phase appears to determine the number of follicular waves during an estrous cycle (Fortune 1994)

Fertility may be regulated by the number of follicular waves during the estrous cycle Heifers with three waves had higher P_4 concentrations at the time luteolysis would occur and this may antagonize the uterine luteolytic mechanism Reproductive management systems to optimize conception rates should take in account the possibility of increasing P_4 levels or the numbers of waves within the

estrous cycle as an approach to increase fertility The effects of higher P_4 concentrations at the time of luteolysis or an effect of P_4 on the oocyte that will be ovulated need further investigation

Functional dominance is regulated by a combination of endocrine paracrine and autocrine factors that permit the dominant follicle to exert interovarian intraovarian and intrafollicular effects. Clearly treatment with hCG or LH terminated functional dominance of the first wave follicle due to its induced ovulation Following the loss of functional dominance a newly recruited follicular wave occurred much earlier than the spontaneous second wave follicle of the control group Additional evidence for endocrine regulation of follicle development was the decrease in both morphological dominance in which the second wave follicle did not grow as large and functional dominance in which turnover of the second wave follicle and emergence of the third wave occurred earlier compared to the control group These effects were attributed to higher feedback inhibition of progesterone on LH secretion

Ovarian status at the time of superovulatory treatment has been postulated as a major factor determining ovarian responses (Monniaux et al 1993) The effects of the presence of a dominant follicle at the time of initiation of a superovulatory regime has been controversial Some researchers (Grasso et al 1989 Guilbault et al 1991 Savio et al , 1991 Huhtinen et al 1992 Bungartz and Niemann 1994 Wolfsdorf et al 1997) found that initiation of a superovulatory regime in the absence of a dominant follicle is beneficial However others (Gray et al. 1992 Rajamahendran and Caldar 1993 Stock et al 1993) did not find

differences in yield of transferable or total number of embryos when FSH treatment began during the period of morphological regression (Gray et al 1992) or after ovulation of the dominant follicle (Rajamahendran and Calder 1993, Stock et al 1993). In the experiment described in Chapter 4, there were no differences in response to FSH induction of superovulation between groups with a persistent functionally dominant follicle present and the group with a persistent functionally dominant follicle that was ablated by aspiration during treatment. Numbers of ovulatory follicles, CL, and numbers of total and transferable embryos did not differ between groups.

The persistent dominant follicle model is an alternative to study the effects of a dominant follicle during follicular development (Sirois and Fortune 1990, Savio et al 1993b). In this study (Chapter 4), presence and duration of the dominant follicle were experimentally controlled through induction of a persistent dominant follicle. A similar model was utilized by Wehrman et al (1996), who found a reduced number of CL, total ova and transferable embryos when FSH treatment started 5 days after creation of a persistent dominant follicle. In the present experiment, the dominant follicle was present in the ovary until day 10 of the estrous cycle, and the FSH treatment began 2 days after aspiration of the dominant follicle. The persistent dominant follicle inhibited recruitment in the control group, whereas in the group without the dominant follicle, recruitment occurred 2 days earlier. Likewise, number of class 3 follicles increased earlier in the treated group. This difference in follicular dynamics indicates that there is a factor secreted by the dominant follicle that inhibits recruitment and growth of follicles on both ovaries.

However this effect can be overridden with continued injection of high doses of FSH over a 3 day period Secretion of inhibins may be the inhibitory component but such an affect would likely be at the intercvarian level since any decrease in basal FSH concentrations would have a minimal affect compared to the large doses of FSH (3 to 5 mg) injected over a 3 day period

There is additional evidence that a factor (s) secreted by the dominant follicle influences growth of subordinate follicles in the cohort. Passive immunization of ewes (O Shea et al 1991 Schanbacher et al 1991) or heifers (Price et al., 1987, Glencross et al 1992 Morris et al 1993 Scanlon et al., 193) against steroid free-follicular fluid or against synthetic inhibin peptides increased ovulation rates Follicular dominance appears to be controlled by a number of mechanisms acting in concert Decreases in spontaneous FSH concentrations in plasma is a consequence of E_2 exerting a negative feedback on FSH secretion and the action of inhibin The decrease in FSH may decrease development of subordinate follicles (Ko et al 1991, Adams et al 1992a Fortuna 1994) Secretion of local intrafollicular paracrine factors also may inhibit development of subordinate follicles (Campbell et al , 1995 Armstrong and Webb 1997) and play a role in manifesting suppressed follicular growth of subordinate follicles during the dominance phase

It has been shown that inhibin-like molecules have regulatory actions within the ovary that regulate the synthesis of E_2 through the stimulation in synthesis of androstenedione a precursor for E_2 synthesis (Hillier et al., 1991b Woodruff et al., 1990 Wrathall and Knight 1993) In addition to inhibin stimulating androstenedione production by thecal cells perhaps inhibin may alter E_2 synthesis by granulosa cells

In order to test the latter hypothesis an *in vitro* system for the culture of granulosa cells to produce E_2 was developed (Chapter 5)

Granulosa cells through the secretion of E_2 influence the endocrine status of the female (Skinner and Osteen 1988) Granulosa cells are the follicular component that synthesize and secrete E_2 by conversion of androgens into estrogens Within the follicle androgens are provided by theca cells to be aromatized into estrogens by granulosa cells In the literature there is considerable controversy regarding the ability to maintain E_2 secretion in bovine granulosa cells cultured in vitro In most cases granulosa cells luteinize in culture when exposed to high doses of FSH (Bemdtson et al 1995 Kuran et al 1996) The system validated in Chapter 5 indicated that antral bovine granulosa cells are estrogenic after 96 h in culture but E_2 production depended upon the type of FSH used to stimulate the cells The response of antral bovine granulosa cells depended upon the type of FSH used to stimulate the cells A highly purified porcine FSH preparation (CY-FSH) was able to stimulate granulosa cells to produce E_2. The production of E_2 decreased with time in culture in agreement with others (Bemdtson et al 1995 Rouillier et al 1996) and most likely reflects a reduction in the activity of the aromatase enzyme (Skinner and Osteen 1988 Rouillier et al 1996) The response of granulosa cells to different doses of CY-FSH varied with time At 48 h of culture granulosa cells responded to low doses of FSH (e g 0 5 1 and 2 ng/ml) This ability was lost after 72 h and higher doses of CY-FSH (e g 6 8 and 10 ng/ml) were necessary to stimulate an estradiol response In contrast, a dose of 50 ng/ml of CY-FSH elicited an inhibitory E_2 response Perhaps this reduction

was due to a down regulation of the FSH receptor Cells treated at 48 h with CY FSH had a quadratic E_2 secretory dose response to CY-FSH with a maximal response at 2 ng/ml of CY FSH

Ovine (Langhout et al 1991 Spicer an Alpizar 1994 Berndtson et al 1995 Kuran et al 1995 1996 Spicer and Stewart, 1996) porcine (Saumande 1991 Rouillier et al 1996) and bovine (Gutiérrez et al 1997) FSH preparations have been used to stimulate granulosa cells in culture In the present study responses to ovine and porcine FSH were different but also the response to the porcine CY-FSH from France was greater than the porcine FSH from Belgium These variations are possibly due to presence of other proteins (e g presence of BSA as a carrier in the USDA-ovine FSH preparation) that could affect the biological action of FSH on granulosa cells or BSA itself stimulating luteinization and proliferative responses due to presence of growth factors and fatty acids in the BSA Also FSH exists as a family of isohormones with differences in their oligosachande structures including the degree of sialylation and/or sulfation (Ulloa-Aguirre et al 1995) These differences alter the metabolic clearance rates the biological and immunological potencies and the ability of the isoforms to bind the FSH receptor (Ulloa-Aguirre et al 1984) Perhaps differences in oligosachande structure among the different sources of FSH utilized in this experiment (Chapter 5) can account for differences in their ability to bind the FSH receptor and elicit an estrogenic response by bovine antral granulosa cells in culture Differences in tertiary structure may have had differential effects on sensitivity to lyophylization and subsequent biological activity

Bovine antral granulosa cells did not show a clear response to different

doses of FSH in terms of P_4 secretion. Granulosa cells tend to luteinize with time in culture (Skinner and Osteen, 1988). However, cells treated with CY-FSH secreted decreasing amounts of P_4 per cell with time in culture, whereas preparations of B FSH and USDA-FSH increased P_4 production by granulosa cells.

Shape and number of the granulosa cells was influenced by the different sources of FSH. Cells treated with CY-FSH had a round shape and grew in multilayered aggregates, as reported for the rat (Knecht et al., 1981) and cow (Gutiérrez et al., 1997). However, granulosa cells treated with B FSH and USDA-FSH grew in monolayers and assumed a fibroblastic appearence. The number of cells decreased with time in culture. At 48 h there was an appreciable loss in cell numbers for all FSH treatments with fewer cells at each time compared to number of cells seeded. The ability of cells to remain plated is probably related to the shape they assume in culture. Round cells have less area of contact with the plate, hence cells are more prone to be lost with changes in culture medium at each 24 h period. Skinner and Osteen (1988) reported that granulosa cells isolated from large follicles, as is our case, had a slightly lower plating efficiency and a greater loss of cells between 3 and 6 days of culture. Although cell numbers increased with time for USDA-FSH, it was not determined whether this increase reflected true proliferation or less cell loss. In recent reports, bovine (Gutiérrez et al., 1997) and ovine granulosa cells (Campbell et al., 1996) were shown to proliferate in culture.

Based on the ability of bovine antral granulosa cells to secrete estradiol in response to CY-FSH in culture, it was considered that this was an appropriate *in*

vitro experimental model to examine potential regulatory factors from the dominant follicle that alter E_2 secretion of granulosa cells

Emergence of a follicular wave is controlled by various factors. Follicle-stimulating hormone is responsible for the emergence of each follicular wave. Once the dominant follicle is selected and starts to secrete increasing amounts of E_2, circulating concentrations of FSH decrease, which suggests that the dominant follicle secretes a factor (s) that in an endocrine manner decreases secretion of FSH from the pituitary (Figure 7.1). It is known that inhibin is a slow but powerful inhibitor of pituitary FSH biosynthesis and secretion (Gaddy-Kurten et al., 1995). It also has been suggested that an α inhibin form can bind to FSH binding sites on granulosa cells (Sato et al., 1982; Schneyer et al., 1991) and inhibit the stimulatory action of FSH.

In addition, there are changes in amounts and proportions of the different molecular forms of inhibin (de la Sota, 1995; Sunderland et al., 1996) and expression of inhibin/activin mRNA for α, β_A and β_B subunits (Ireland and Ireland, 1994) that are related to physiological stages of follicle growth (Guilbault et al., 1993; de la Sota, 1995; Sunderland et al., 1996). Dominant follicles have different amounts of monomeric and dimeric forms of inhibin according to their stage of development. De la Sota (1995) reported differences between day 5 DF and day 12 ADF in amounts and proportions of monomeric and dimeric forms of inhibin. Based on these findings and the hypothesis that inhibin can bind to the FSH receptor of granulosa cells, the experiment in Chapter 6 examined the effect of follicular fluid complete from day 5 dominant and day 12 atretic dominant follicles

and respective follicular fluid fractions prepared from an antiinhibin immunoaffinity column on E_2 secretion by bovine antral granulosa cells

It is reasonable that regulatory factors produced by dominant follicles would be present in the follicular fluid. It is probable that large molecular weight factors such as the dimeric forms of inhibin (≥ 34 kDa) in follicular fluid were produced by granulosa cells since the basement membrane would separate thecal cells from granulosa cells. Thus factors within the follicular fluid would represent an array of molecules that are candidates for intrafollicular regulation of granulosa cells and interovarian regulators if secreted from the ovary. One family of factors may be the inhibin family of proteins described in cattle (Good et al., 1995; de la Sota, 1995). Utilizing the antiinhibin immunoaffinity column prepared as described in Chapter 5, two regulatory components of follicular fluid could be prepared. The inhibins-eluate represents the family of inhibin proteins, and the follicular fluid eluate without inhibins represents the remaining factors in follicular fluid that may potentially regulate estradiol secretion by granulosa cells. The preparation of the follicular fluid pools prior to fractionation eliminated the presence of steroids such as estradiol and progesterone.

Results from granulosa cell cultures (Chapter 6) showed that follicular fluid from day 5 DF appears to have factors that inhibit E_2 secretion. These factors are present in the fraction without inhibins and in the inhibin-enriched fraction. In the follicular fluid fraction without inhibins, other factors may be present such as TGF-α (Rouillier et al., 1997), IGFBPs (Monget and Monniaux, 1995), tissue inhibitor of metalloproteinase (Rocha, 1996), and hsp90 (Roche, 1995; Dinsmore, personal

communication) that may inhibit E_2 secretion. The inhibin-enriched fraction had a 49 kDa form of immuno-reactive inhibin that could be responsible for the inhibition of E_2 secretion. This 49 kDa form was shown to be a monomeric α form (Good et al. 1995; Sunderland et al. 1996) and may bind to the FSH receptor since has been shown that α- inhibin can bind to FSH binding sites on rat granulosa cells (Schenayer et al., 1991). These inhibitory effects were not observed with a dose of 0.5 ng/ml of CY-FSH. Perhaps these inhibitors in follicular fluid without inhibin and inhibins-eluates compete for the FSH receptor or attenuate components of the signal transduction system to inhibit aromatase activity and E_2 secretion. In day 12 follicular fluid fractions, the inhibins-eluate fraction of the day 12 ADF had an opposite stimulatory effect on E_2 secretion. However, the follicular fluid without inhibins fraction inhibited E_2 secretion by the granulosa cells. Proportions of the mature 34 kDa form are high in follicular fluid of day 12 ADF (de la Sota., 1995; Sunderland et al., 1996) and it has been hypothesized (Baird and Smith, 1993) that increased secretion of E_2 is in part dependent on the stimulation by inhibin of LH-induced androgen synthesis by theca cells. However, this explanation may not apply to the present experimental results because the culture system lacks the thecal component of the follicle. A direct stimulatory effect of the inhibins-eluate the FSH signal transduction system leading to an increase in aromatase enzyme activity.

Progesterone secretion was decreased by 10 ng/ml of CY-FSH. The higher E_2-P_4 ratio in culture media from granulosa cells treated with 10 ng/ml of CY-FSH

indicated that granulosa cells had a higher estrogenic potential This effect was present regardless of whether the fractions were from day 5 DF or day 12 ADF

In summary the effect of the dominant follicle is exerted at different levels At an endocrine level inhibin decreases synthesis and secretion of FSH from the pituitary (Figure 7-1) At the interovarian level secretion of an endocrine factor (e g. inhibin forms) act on both ovaries to alter E_2 secretion (Figure 7-1) and at the intraovarian level through the action of inhibin forms within the follicle on granulosa cells to alter E_2 (Figure 7-2 and 7-3) These factors appear to work in an orchestrated manner in order to control follicular development

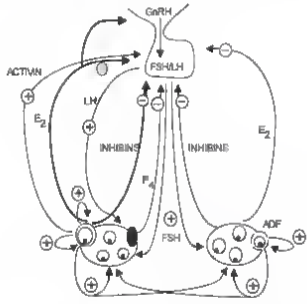

Figure 7 1 Endocrine and iterovarian regulation of estradiol secretion

Estradiol and inhibin complement each other in regulating the secretion of FSH Both hormones secreted in higher amounts by the dominant follicle and

lesser amounts by subordinate follicles exert a negative feedback on the hypothalamus (E_2) and hypophysis (inhibin) to decrease FSH release (Baird and Smith 1993) In addition to E_2 and inhibin the dominant follicle also secretes activin and follistatin Activin stimulates the secretion of FSH from the pituitary whereas follistatin acts through the binding of activin to abolish the stimulatory effect of activin Therefore the decrease in FSH secretion compromises development of subordinate follicles During the luteal phase the corpus luteum secretes progesterone which exerts a negative feedback on the secretion of LH from the adenohypophysis The decrease in concentrations of LH negatively affects the development of the dominant follicle leading to atresia (Sirois and Fortune 1990 Savio et al 1993b)

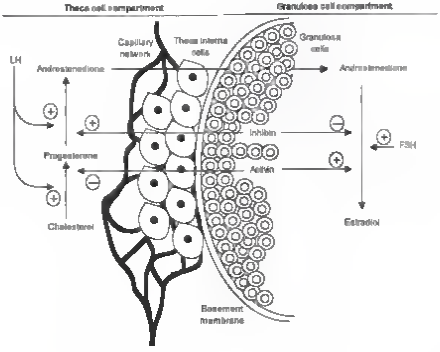

Figure 7-2 Intrafollicular and interovarian effects of inhibin on estradiol secretion

The interovarian effect of the dominant follicle appears to be through the action of inhibin in an endocrine manner. Follicle-stimulating hormone acts on the granulosa cells of the dominant follicle to induce aromatase activity, inhibin production and LH receptors. Inhibin secreted by the dominant follicle acts on subordinate follicles within the same ovary and the contralateral ovary (Figure 7-2). The action of inhibin on subordinate follicles may be change the regulation of E_2 synthesis. It has been shown that inhibin stimulates the LH-induced synthesis of androgen by theca cells (Findlay, 1993; Hillier et al., 1991; Wrathall et al., 1995). Activin prevents luteinization through a negative effect on progesterone synthesis (Findlay, 1993) by the theca cells and stimulatory effects on E_2 synthesis in granulosa cells (Baird and Smith, 1993; Figure 7-2).

A potential model that could explain the regulatory mechanisms at the level of the granulosa cells that control dominance in cattle is depicted in Figure 7-3. When FSH binds to its receptor on the granulosa cell, there is an activation of the cAMP/protein kinase A and other cAMP-dependent pathways. As a consequence, there is stimulation of the aromatase enzyme, that synthesizes estrogens from thecal androgens. The inhibitory factors (e.g., an inhibin form) secreted by the dominant follicle could bind to the FSH receptor on the granulosa cell and uncouple the FSH signal transduction system. This would lead to a decrease in aromatase activity due to decreased gene expression of aromatase and decreased aromatase activity in the dominant follicle. If this factor is secreted it has the ability to act on subordinates follicles in a comparable manner to decrease follicle growth and E_2 secretion. There are other factors (e.g., TGF-α, IGFBPs, hsp90) that are secreted

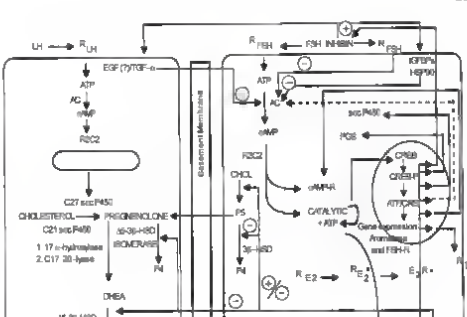

Figure 7-3 Regulation of steroidogenesis in thecal and granulosa cells

into the follicular fluid which may affect the granulosa cell physiology The secretion of these factors may interact with inhibin forms to alter E_2 secretion For example the stimulatory effects of inhibins-eluate in day 12 follicular fluid on E_2 secretion was not evident when mixed with the fraction of follicular fluid without inhibins that contained inhibitory components to E_2 secretion Such a mixture was considered to be comparable to follicular fluid complete which inhibited E_2 secretion This is not to be unexpected when it is known that atretic follicles have high concentrations of IGFBP-2 -4 and -5 (de la Sota 1995) that may sequester IGF-I and IGF-II and

prevent their stimulatory effects on granulosa cells (e.g., positive effects on aromatase). It has been shown (Legault et al. 1997) that TGF-α inhibits FSH induced E_2 secretion of granulosa cells in culture. This is done through the activation of protein kinase C pathway that inhibits the formation of cAMP.

In summary, several factors interplay to control follicular development in cattle. These factors act in an endocrine, interovarian, intraovarian and intrafollicular fashion. One of the principal and strongest candidates are the different inhibin forms secreted by the dominant follicle. Whereas the dimeric forms are important in the control of the FSH release by the anterior pituitary, some of the α-inhibin forms have been postulated to act at the level of the granulosa cells, inhibiting the binding of FSH to its receptor.

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BIOGRAPHICAL SKETCH

Thais del Valle Diaz Zambrano was born September 15, 1957, to Maria Antonia and Francisco Diaz in Lagunillas, Zulia State, Venezuela. She is one of six children. She was accepted as a student at the Veterinary Sciences College of Universidad Central de Venezuela in 1975 where she received the degree of Médico Veterinario (Venezuelan equivalent of D.V.M.) in November 1980. During the same year she started her Masters under the supervision of Dr. Magaly R. de Manzo. Three years later, November 1983, she received the degree of Magister Scientiarum from Universidad Central de Venezuela. After graduation, she started to work as Assistant Researcher at the Veterinary College of Universidad Central de Venezuela. In 1986 she was accepted at the Veterinary College as Instructor Professor in the department of Animal Production. In 1990 she became Assistant Professor, which is her current position. In August 1992, she began her doctoral program under the supervision of Dr. William W. Thatcher. After completing the degree of Doctor of Philosophy, she will return to Venezuela where she will resume her position at the Veterinary College of Universidad Central de Venezuela.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy

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May, 1998

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